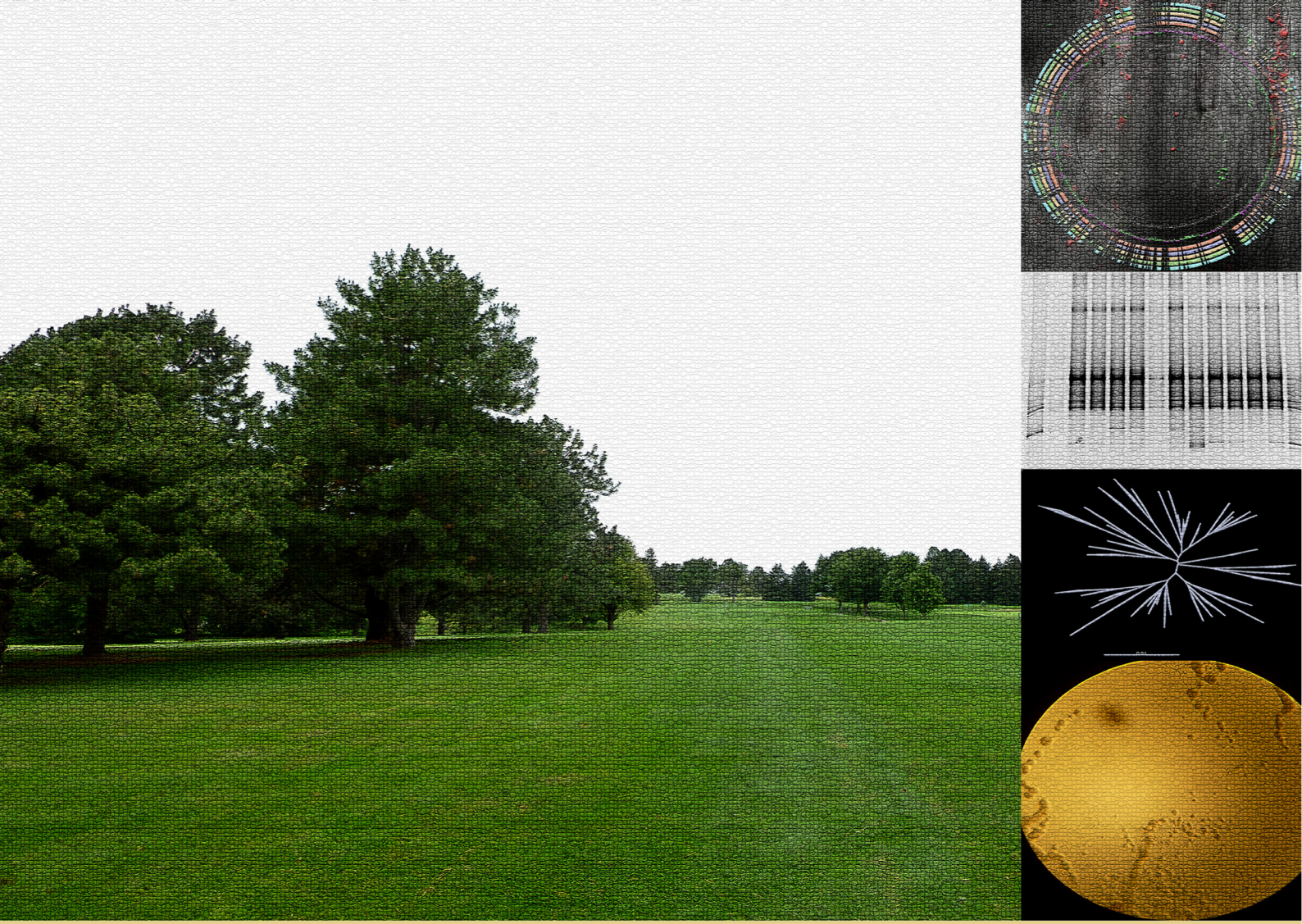

Diogo Alexandre Neves Proença

ROLE OF ENDOPHYTIC MICROBIAL COMMUNITY IN PINE WILT DISEASE

Tese de Doutoramento em Bioquímica, especialidade de Microbiologia, orientada pela Senhora Professora Doutora Paula Maria de Melim Vasconcelos de Vitorino Morais e pelo Senhor Professor Doutor Gregor Boris Grass e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

2014

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UNIVERSIDADE DE COIMBRA

Departamento de Ciências da Vida
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Front cover: Brief overview of all work presented in this thesis. Landscape with healthy pine trees on left. On right, from top to bottom: colonization of *Serratia* sp. M24T3 in *Arabidopsis thaliana* and its genome sequence circular map; DGGE profiling; phylogenetic tree with all endophytic bacterial sequences from this study; and bacterial colonies from pinewood nematode on agar plates.

Tese apresentada ao Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra para prestação de provas de Doutoramento em Bioquímica, especialidade de Microbiologia, realizada sob a orientação científica da Senhora Professora Doutora Paula Maria de Melim Vasconcelos de Vitorino Morais, Professora Auxiliar do Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, e do Professor Doutor Gregor Boris Grass, Investigador Sénior no *Bundeswehr Institute of Microbiology*, Munique, Alemanha.

O trabalho que levou à presente tese foi realizado no Instituto do Mar, Universidade de Coimbra, Coimbra, Portugal, em *School of Biological Sciences, University of Nebraska-Lincoln*, Lincoln, Estados Unidos da América, e na Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brasil.

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À Sara

À Rita

Aos meus pais

Às minhas irmãs

This is where humanity's paths part:

if you want peace of soul and happiness,

then believe,

but if you want to be a follower of truth,

then seek.

(Brief an Elisabeth Nietzsche (11 June 1865),

in: Werke in drei Bänden, München, 1954, 953ff.)

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Table of Contents

Abstract

Pine wilt disease (PWD), a major illness of several *Pinus* species, native to North-America, has spread into Asia and recently into Europe. The first report in Portugal was in 1999 but since 2008 has spread to the Center-North of the country. *Bursaphelenchus xylophilus*, the pinewood nematode (PWN), is considered the only causative agent of PWD. In recent years, it has been proposed that PWD is a complex disease induced by both PWN and the bacteria it carries. The present thesis aimed to assess the microbial community, within the host-plant and associated with the nematode upon nematode infection in PWD, in order to elucidate the community structure and to understand roles of several bacteria involved. The endophytic microbial community structure was studied from infected and non-infected pine trees, *Pinus pinaster*, in Avô and Malhada areas, based on culture isolates and molecular profiling (denaturing gradient gel electrophoresis - DGGE). The endophytic strains were identified by 16S rRNA gene sequencing. The classes *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria*, *Flavobacteria*, *Gammaproteobacteria* and *Sphingobacteriia* were identified from the sampling areas. *Gammaproteobacteria* were the most abundant bacteria. DGGE profiles failed to produce a common pattern for PWD indicating that the microbial community was diverse and variable within diseased pine trees. DGGE detected the presence of endophytes belonging to six additional classes: *Bacteroidia*, *Deinococci*, *Fusobacteria, Spirochaetes, Planctomycetaceae* and *Verrumicrobiae*. Furthermore, Archaea were found as part of the endophytic community (DGGE), belonging to the phyla *Euryarchaeota, Thaumarchaeota* and *Crenarchaeota*. Finally, the presence of *nifH*, *nifJ*, *nirS* and *nirK* genes in the endophytic bacterial community supports the possibility of N_2 fixation inside the trees, and also denitrification, with the production of the metabolic relevant intermediary NO. The presence of Bacteria and Archaea as part of the endophytic community in *P. pinaster* could potentiate the ability of pine trees to adapt to changes in the habitat.

An endophytic bacterium was characterized by a polyphasic approach and described as new species *Chitinophaga costaii* A37T2^T. The diversity of PWN-associated bacteria in Portugal and USA was assessed in this work. The results obtained were compared with information from databases, in order to detect specific bacterial species associated with the nematode, and to make suggestions on the role of these bacteria in PWD. In Portugal, the associated bacteria were studied in the previously mentioned two sampling areas and in the first area where PWN was identified in this country, Setúbal. All isolates, except one Gram-positive strain (*Actinobacteria*), belonged to the

Gram-negative *Beta-* and *Gammaproteobacteria*. Most isolates belonged to the genus *Pseudomonas, Burkholderia* or to the family *Enterobacteriaceae*. The strains were able, depending on sampled areas, to produce siderophores (60-100%), proteases activity (0-30%), and lipases activity (10-100%). This suggests that the ability to produce siderophores or lipases by most isolates may enable these bacteria to play a role in plant physiological response to external factors. In the USA, bacteria carried by PWN mainly belonged to the class *Gammaproteobacteria.* The genera *Chryseobacterium* (class *Flavobacteriia*) and *Pigmentiphaga* (class *Betaproteobacteria*) were for the first time found associated with nematodes in the USA. The major bacterial population associated with the nematodes differed depending on the forest area/country and none of the isolated bacterial species was found in all different forest areas. Only strains from the genus *Pseudomonas* were found carried by nematodes from all countries.

Moreover, the potential nematotoxic activity of bacteria carried by PWN was tested. Twenty-one strains showed capacity to produce extracellular products with nematicidal activity. The strain with the highest nematicidal activity, *Serratia* sp. A88copa13, produced proteases in the supernatant and a serine protease with 70 kDa was identified as the major factor responsible for the toxicity against PWN and other *Bursaphelenchus* species.

The genome of two bacterial strains, *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1, with nematotoxic activity was sequenced, in order to identify genes potential involved in toxicity towards PWN. The genome sequence of both strains featured genes that might be involved in nematotoxicity. In addition, potentially plant-growth promoting bacterial genes were also found in both genome sequences. The colonization process in *Arabidopsis thaliana* by *Serratia* sp. M24T3, labeled with *gfp* and *gusA* reporter genes, was evaluated and the identification genes potentially involved in plant growth promotion was investigated. Strain M24T3 showed potential to be a plant growth-promoting bacterium, since it produced siderophores, solubilized phosphate and zinc oxide and because it has a 1-aminocyclopropane-1-carboxylate deaminase gene, it most probably lowers plant ethylene levels. Findings with *A. thaliana* demonstrated *Serratia* sp. M24T3 to be able to colonize other plants beyond pine trees.

In conclusion, the results presented in this thesis provided a broad overview and comprehensive insight of the microbial diversity and functionality related to PWD.

Keywords: Archaea, Bacteria, diversity, DGGE, Pine wilt disease, *Bursaphelenchus xylophilus*, *nif*, *nir*, *Pinus pinaster*, *Arabidopsis thaliana*, *Serratia*, *Pseudomonas*, genome.

Resumo

A doença da murchidão do pinheiro (DMP), uma das principais doenças de várias espécies de *Pinus*, nativa da América do Norte, espalhou-se para a Ásia e, recentemente, na Europa. O primeiro registo em Portugal foi em 1999, mas em 2008 alastrou para o Centro-Norte do país. *Bursaphelenchus xylophilus*, o nemátode da madeira do pinheiro (NMP), é considerado o único agente causador da DMP. Nos últimos anos, tem sido proposto que a DMP é uma doença complexa induzida por ambos, o NMP e as bactérias a ele associadas. A presente tese teve como objectivo avaliar a comunidade microbiana, dentro da planta hospedeira e associada ao nematode, após infecção pelos nemátodes na DMP, a fim de elucidar a estrutura da comunidade e compreender a função das várias bactérias envolvidas. A estrutura da comunidade microbiana endofítica foi estudada a partir de pinheiros infectados e não infectados, *Pinus pinaster*, nas áreas de Avô e Malhada, com base no cultivo de isolados e no perfil molecular (electroforese em gel de gradiente desnaturante - DGGE). As estirpes endofíticas isoladas foram identificadas por sequenciação do gene 16S rRNA. Estirpes das classes *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria*, *Flavobacteria*, *Gammaproteobacteria* e *Sphingobacteriia* foram identificadas nas áreas de amostragem. *Gammaproteobacteria* foi a classe que apresentou maior número de isolados. Os perfis de DGGE da comunidade microbiana não apresentaram um padrão comum em pinheiros com DMP, indicando que a comunidade era diversa e variável dentro destes. DGGE detectou a presença de endófitos pertencentes a seis classes adicionais: *Bacteroidia*, *Deinococci*, *Fusobacteria, Spirochaetes, Planctomycetaceae* e *Verrumicrobiae*. Além disso, foram encontradas Archaea como parte da comunidade endofítica (DGGE), pertencente aos filos *Euryarchaeota, Thaumarchaeota* e *Crenarchaeota*. Finalmente, a presença dos genes *nifH*, *nifJ*, *nirS* e *nirK* na comunidade endofítica bacteriana suporta a possibilidade de fixação de N_2 no interior das plantas, e também de desnitrificação, com a produção de NO intermediário metabólico relevante. A presença de Bacteria e Archaea como parte da comunidade endofítica em *P. pinaster* poderá potenciar a capacidade de adaptação dos pinheiros às mudanças no habitat.

Uma das bactérias endofíticas foi caracterizada por uma abordagem polifásica e descrita como nova espécie *Chitinophaga costaii* A37T2^T. A diversidade de bactérias associadas ao NMP em Portugal e nos EUA foi avaliada neste trabalho. Os resultados

obtidos foram comparados com a informação contida nas bases de dados, a fim de detectar as espécies bacterianas associadas ao nemátode, e sugerir o papel destas na DMP. Em Portugal, foram estudadas as bactérias associadas das duas áreas de amostragem mencionadas anteriormente e na primeira área onde NMP foi identificado neste país, Setúbal. Todos os isolados, excepto uma estirpe Gram-positiva (*Actinobacteria*), pertenciam a Gram-negativos *Beta-* e *Gammaproteobacteria*. A maioria dos isolados pertencia aos géneros *Pseudomonas*, *Burkholderia* ou à família *Enterobacteriaceae*. As estirpes foram capazes, dependendo das áreas amostradas, de produzir sideróforos (60-100 %), ter actividade proteolítica (0-30 %) e actividade lipídica (10-100 %). Isto sugere que a capacidade de produzir sideróforos ou lípases, observada na maioria dos isolados, pode permitir que estas bactérias desempenhem um papel na resposta fisiológica da planta a factores externos. Nos EUA, as bactérias transportadas pelo NMP pertenciam principalmente à classe *Gammaproteobacteria*. Os géneros *Chryseobacterium* (classe *Flavobacteriia*) e *Pigmentiphaga* (classe *Betaproteobacteria*) foram pela primeira vez encontrados associados com nemátodes nos EUA. A população bacteriana maioritária associada aos nemátodes diferiram dependendo da área florestal/país e nenhuma das espécies bacterianas isoladas foi encontrada em todas as áreas florestais diferentes. Somente estirpes do género *Pseudomonas* foram encontradas como transportadas pelos nemátodes de todos os países.

Além disso, foi testado o potencial da actividade nematotóxica das bactérias transportadas pelo NMP. Vinte e uma estirpes mostraram capacidade para produzir produtos extracelulares com actividade nematicida. A estirpe com maior actividade nematicida, *Serratia* sp. A88copa13, produziu protéases no sobrenadante e uma protéase serínica com 70 kDa foi identificada como o principal factor responsável pela toxicidade contra o NMP e outras espécies de *Bursaphelenchus*.

Os genomas das duas estirpes bacterianas, *Serratia* sp. M24T3 e *Pseudomonas* sp. M47T1, com actividade nematotóxica foram sequenciados, a fim de identificar potenciais genes envolvidos na toxicidade para NMP. A sequência do genoma de cada uma das estirpes apresentou genes que podem estar envolvidos na nematotoxicidade. Além disso, potenciais genes bacterianos de promoção de crescimento de plantas foram encontrados em ambas as sequências do genoma. O processo de colonização em *Arabidopsis thaliana* pela *Serratia* sp. M24T3, marcada com os genes repórteres *gfp* e

gusA, foi avaliado e a identificação de genes potencialmente envolvidos na promoção do crescimento das plantas que coloniza foi investigada. A estirpe M24T3 mostrou potencial para ser uma bactéria promotora do crescimento da planta, uma vez que produziu sideróforos, solubilizou fosfato e óxido de zinco e porque tem um gene de desaminase de 1-aminociclopropano-1-carboxilato, que muito provavelmente reduz os níveis de etileno das plantas. Os resultados obtidos com *A. thaliana* indicam que *Serratia* sp. M24T3 é capaz de colonizar outras plantas além pinheiros.

Em conclusão, os resultados apresentados nesta tese proporcionaram uma visão ampla e abrangente da diversidade microbiana e funcionalidade relacionada à DMP.

Palavras-chave: Archaea, Bacteria, diversidade, DGGE, Doença da murchidão do pinheiro, *Bursaphelenchus xylophilus*, *nif*, *nir*, *Pinus pinaster*, *Arabidopsis thaliana*, *Serratia*, *Pseudomonas*, genome.

Thesis outline

This thesis presents a broad extension on our understanding of the role of the endophytic microbial community in pine wilt disease (PWD), through analysis of the diversity of the endophytic community itself and the bacteria associated with the pinewood nematode (PWN) from different countries. This work further includes descriptions of new species, investigations of nematicidal activity exerted by bacteria, insights into bacterial genomics and plant-microbe interactions.

The thesis is divided into General Introduction (Chapter 1), Chapters 2-8 featuring results from original research and Concluding remarks (Chapter 9) highlighting the major research findings. The structural and functional diversity of the microbial endophytic community in pine trees affected with PWD was assessed in Chapter 2. A new endophytic species of the genus *Chitinophaga* was described in Chapter 3. The diversity of bacteria carried by the pinewood nematode (PWN) *Bursaphelenchus xylophilus*, from different countries, was assessed in Chapters 4 and 5 in order to understand the potential role of bacteria in PWD. The nematicidal properties of the bacteria carried by PWN were included in Chapter 6. Genome sequences of two bacterial isolates carried by PWN that have nematicidal activity were presented in Chapter 7. Plant-bacteria interactions, plant colonization and effects on plant growth by bacteria, previously isolated (Chapter 4), with positive nematotoxic activity (assessed in Chapter 6) and genome sequenced (Chapter 7), were further characterized in Chapter 8.

The detailed outline of Chapters 2-8 is as follows:

In **Chapter 2**, the structure of the endophytic microbial community was assessed in Portuguese pine trees. Cultivation and molecular methods were used to understand the microbial community present inside pine trees. As microbial communities have different roles depending on their different ecological niches, functional diversity based on *nifH*, *nifJ*, *nirK* and *nirS* genes was also found.

In **Chapter 3**, a new species – *Chitinophaga costaii* – was isolated from the endophytic microbial community of a *Pinus pinaster* tree trunk reported in Chapter 1 and was further characterized. The genus *Chitinophaga* belongs to the family *Chitinophagaceae*,

phylum *Bacteroidetes*. A polyphasic approach based on phenotypic and genotypic information was applied. Moreover, an emended description of *Chitinophaga niabensis* was performed.

In **Chapter 4**, the bacterial diversity carried by PWN and other nematodes from pine trees, from three different affected forest areas in Portugal, was assessed. These bacterial strains showed the production of proteases, lipases and siderophores.

In **Chapter 5**, the bacterial community carried by *B. xylophilus* in the USA, the endemic area of PWD, was isolated and identified in different pine trees species with PWD. Furthermore, in order to identify a bacterial species that might be associated with all *B. xylophilus*, suggesting a privileged relationship, e.g., having a role in PWD, EMBL/GenBank database information and previous publications were included for analysis.

In **Chapter 6**, the potential nematicidal function of the bacterial isolates associated with the Portuguese nematodes, previously identified in Chapter 4, were studied. *In vitro* tests showed a number of isolates were able to kill the nematodes within 24h. The nematicidal activity of strain A88copa13 was related with the ability to produce a 70 kDa serine protease different from those previously describe. The nematotoxic activity of the bacterium differed between species of *Bursaphelencus*.

In **Chapter 7**, *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1 isolated from the nematode *B. xylophilus* in Chapter 4 and showing nematotoxic activity *in vitro* in Chapter 6, were genome sequenced. The draft genome sequences constitute the first step aiming at identifying genes involved in bacterial nematicidal activity in addition to genes with beneficial roles for host plants.

In **Chapter 8**, the use of the plant model *A. thaliana* evaluated the role of nematicidal *Serratia* sp. M24T3, for which nematicidal activity was demonstrated in Chapter 6 and was genome sequenced in Chapter 7, as plant growth-promoting bacterium, based on *in vitro* and *in silico* analysis.

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
AFLP	amplified fragment-length polymorphism PCR
ANME-1	anaerobic methanotrophic archaea
ANOVA	analysis of variance
ARDRA	amplified ribosomal DNA restriction analysis
BA	benzoic acid
BLAST	basic local alignment search tool
BNF	biological nitrogen fixation
BOX-PCR	PCR derived from the *boxA* element
CAA	casamino acids liquid medium
CDS	coding sequence
CLSM	confocal laser scanning microscopy
COG	cluster of orthologous groups
CTAB	cetyl trimethyl ammonium bromide
DBH	diameter of sampled trees was measured at breast height
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMBL	european molecular biology laboratory
EPN	entomopathogenic nematodes
EPPO	European and Mediterranean plant protection organization
EPS	extracellular polysaccharide
ERIC-PCR	enterobacterial repetitive intergenic consensus sequences-PCR
FAME	fatty acid methyl esters
FPLC	fast protein liquid chromatography
GC-FAME	gas chromatographic fatty acid methyl ester
gfp	green fluorescent protein gene
GSR	glutathione-reductase
gusA	β-glucuronidase gene
HGT	horizontal gene transfer
IAA	indole-3-acetic acid
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
ISR	inducing systemic resistance
KEGG	Kyoto encyclopedia of genes and genomes
KO	KEGG orthology
LB	Luria-Bertani medium
LTP	living tree project
MALDI	matrix-assisted laser desorption/ionization
MATE	multidrug and toxin extrusion
MCG	miscellaneous *Crenarchaeote* group
MEGA	molecular evolutionary genetics analysis
MG-I	marine group I
MS medium	Murashige & Skoog medium
MS/MS	tandem mass spectrometry
NA	nutrient agar
NB	Nutrient broth
NCBI	national center for biotechnology information
NGS	next-generation sequencing
nifH	nitrogenase iron protein gene
nifJ	pyruvate:ferredoxin (flavodoxin) oxidoreductase gene
nirK	nitrite reductase gene
nirS	cytochrome cd1 nitrite reductase gene
NO	nitric oxide
OD	optical density
OTU	operational taxonomic unit
PA	phenylacetic acid
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PGAAP	NCBI prokaryotic genomes automatic annotation pipeline
PGPB	Plant growth-promoting bacteria
PGPR	plant growth-promoting rhizobacterium
PMF	peptide mass fingerprinting
PPN	plant parasitic nematodes
PWD	Pine wilt disease
PWN	pinewood nematode
R2A	Reasoner´s 2A medium
RAPD	randomly amplified polymorphic DNA
RAST	rapid annotation using subsystem technology
RAxML	Randomized Axelerated Maximum Likelihood
RDA	redundancy analysis
RDP	ribosomal database project II
REP-PCR	repetitive extragenic palindromic-PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RTX	repeats in the structural toxins
SAGMEG	South African goldmine group
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SINA	SILVA incremental aligner
SOD	superoxide dismutase
ThPP	thiamine pyrophosphate
TOF	time-of-flight mass spectrometer
tRNA	transfer RNA
TSA	tryptic soy agar
UPGAMA	unweighted pair group method using arithmetic averages
VOC	volatile organic compounds

1

General Introduction

1.1. Brief historical overview

Pine wilt disease (PWD) is defined as being caused by the pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Nickle *et al.*, 1981), the only known causal agent of disease. Native to North America (USA and Canada), it was introduced and first reported in Japan at the beginning of the 20th century and it has been spread into China, Korea, Taiwan in the late 1970 – 1980s, and thereafter to Europe (Portugal and Spain) (EPPO/OEPP, 2009; Evans *et al.*, 1996; Fonseca *et al.*, 2012; Mamiya, 1988; Mota *et al.*, 1999; Nickle *et al.*, 1981; Robertson *et al.*, 2011; Sutherland & Webster, 1993; Zhao *et al.*, 2008) (Figure 1.1). Moreover, PWN was also reported in Nigeria and Mexico (Dwinnel, 1993; Khan & Gbadegesin, 1991).

The PWN and *Monochamus* spp. - the insect vector species (see next section 1.2.) - were listed as European and Mediterranean Plant Protection Organization (EPPO) A1 quarantine pests (EPPO/OEPP, 2009), but recently, PWN was re-classified as A2 (http://www.eppo.int/QUARANTINE/quarantine.htm). The purpose of EPPO A1/A2 lists were to regulate phytosanitary concerns of quarantine pests and to point out the attention for plant species in EPPO member countries that pose a threat to plant health, biodiversity and environment. Moreover, the lists distinguish pests which are absent (A1) from those which are present (A2) in the EPPO region.

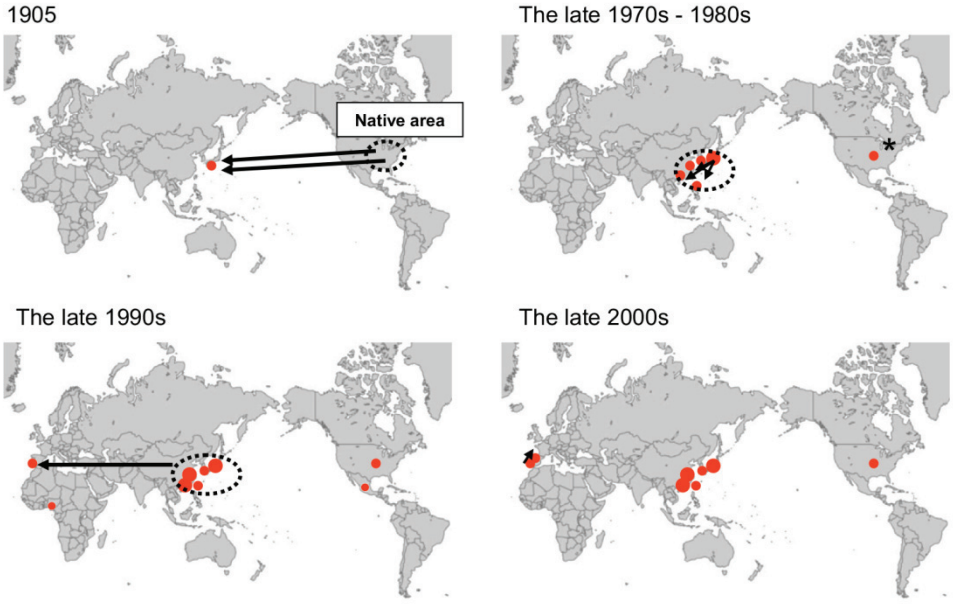


Figure 1.1. Temporal and spatial spreading of pine wilt disease around world. *Occurrence of the disease in forests in North America is mostly limited to non-native tree species. Adapted from Shinya *et al.* (2013).

Usually, the genus *Pinus* is the main host for PWN. However, some species of the genera *Abies*, *Chamaecyparis*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* have also been reported as suitable hosts for PWN and for its life cycle development. Regarding pine trees of the *Pinus* genus, the susceptible species that have been reported are *P. ayacahuite*, *P. densiflora*, *P. kesiya*, *P. koraiensis*, *P. leiophylla*, *P. luchuensis*, *P. mugo*, *P. muricata*, *P. nigra*, *P. pinaster*, *P. sylvestris*, *P. thumbergii* (Evans *et al.*, 1996). The abundance of different *Pinus* species is different according to the forest of each country (Evans *et al.*, 1996). Portuguese forests are mainly comprised by species of pine, eucalyptus and oak trees. In Portuguese pines forest, the most abundant species, maritime pine (*P. pinaster*), is a PWD-susceptible pine species with 62.5 % of all trees, other minor species in such forests are stone pine (*P. pinea*) and Scots pine (*P. sylvestris*) (Rodrigues, 2008).

Therefore, PWN is causing one of the most destructive diseases in trees of the genus *Pinus* and is responsible for environmental and economic losses around the world accruing to tens of million dollars (Tóth, 2011; Vicente *et al.*, 2011a).

1.2. *Bursaphelenchus xylophilus* and PWD development

First *Bursaphelenchus xylophilus* was taxonomy classified as *Aphelenchoides xylophilus* by Steiner & Buhrer, 1934 from blue-stained logs of *P. palustris* at Louisiana, USA but was reclassified by Nickle and coworkers (1981). The genus *Bursaphelenchus* is a member of the phylum Nematoda, class *Secernentea*, order *Aphelenchida* and family *Parasitaphelenchidae* based on phylogenetic analyses (Blaxter *et al.*, 1998).

The transmission of PWN between host trees is mediated by insect vectors. Pine sawyer beetles of the genus *Monochamus* comprise different species, and belong to the family *Cerambycidae* and constitutes the main element in the epidemiology of PWD. In Japan the major vector is *M. alternatus*, in North America *M. carolinensis*, and in Portugal, *M. galloprovincialis* is the only vector (Evans *et al.*, 1996; Linit, 1988; Mamiya & Enda, 1972; Sousa *et al.*, 2001).

The life cycle of the PWN and PWD development is summarized in Figure 1.2. *Monochamus* spp. carry *B. xylophilus* inside their tracheal system and on the body surface. Attracted by healthy trees (primary transmission), the insect vector releases nematodes during feeding on young pine shoots. Whether disease occurs depends on the *Pinus* spp. tree being resistant or susceptible, and in consequence, in the death or

multiplication of the nematodes, respectively. In susceptible pine trees, PWN can feed and multiply in the plant tissues, leading to the death of the host. Moreover, *Monochamus* spp. are also attracted by dead or dying pine trees for ovoposition (secondary transmission). In this instant, there is insect vector larvae development and *B. xylophilus* feed on blue stain fungi (*Grosmannia clavigera*).

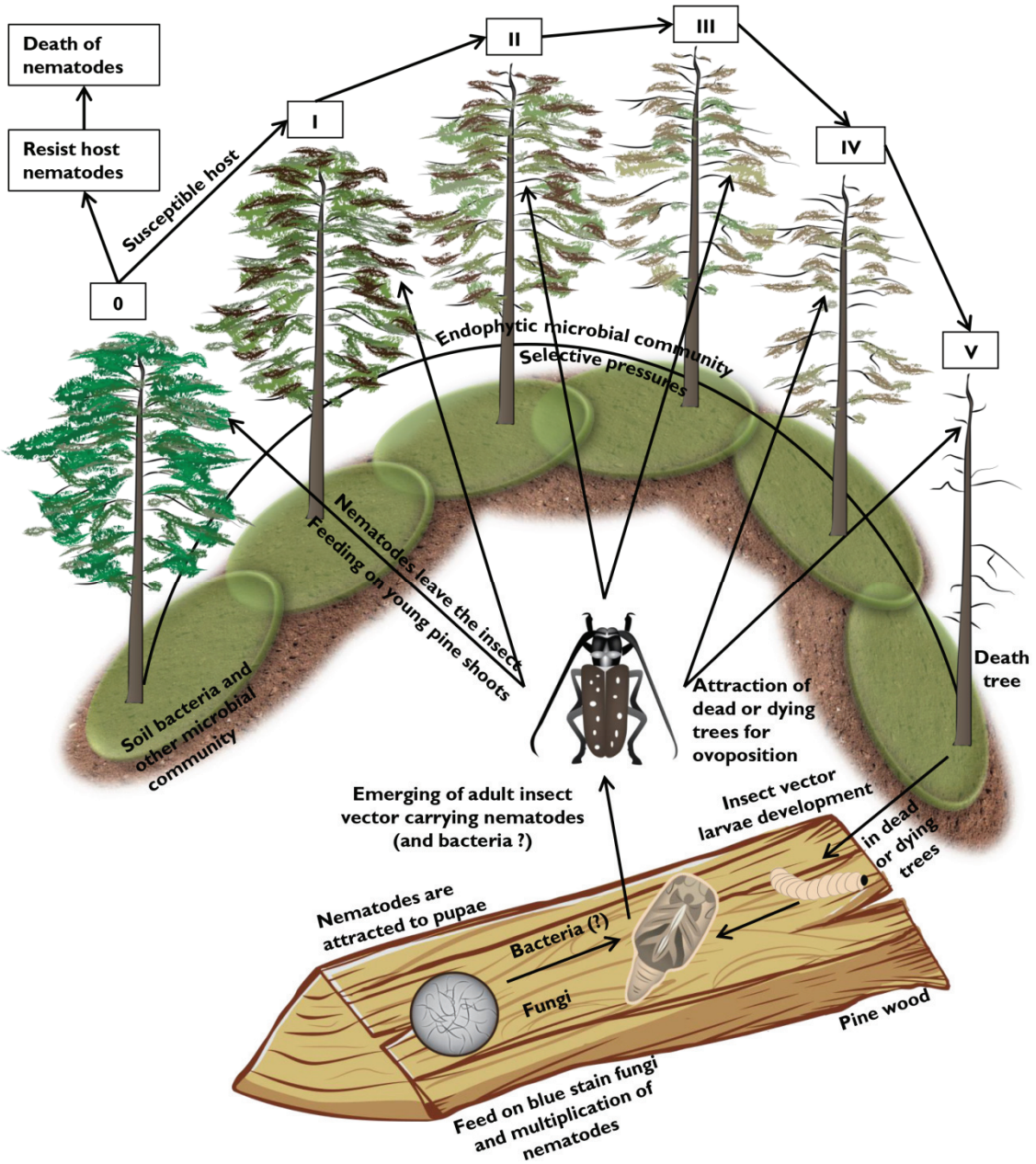


Figure 1.2. Scheme of relationships between *B. xylophilus and Monochamus spp.* life cycles *and* pine wilt disease development. PWD symptom classes: 0 - tree without symptoms; I - ‹10% brown leaves; II - 10-50% brown leaves; III - 50 - 80% brown leaves; IV - › 80% brown leaves; V – dead tree without leaves. Question mark (?) indicates that the bacteria maybe could be carried by nematode into the *Monochamus* spp. but remains unclear.

Before the emerging of the adult insect vector, the nematodes are attracted to pupae, enter their tracheal system and restarts the cycle (Evans *et al.*, 1996; Linit, 1988; Sousa *et al.*, 2001). According to Zhao and coworkers (2007), three terpenes (α-pinene, β-pinene, and longifolene) are produced by larval *M. alternatus* and are able to attract nematodes. Moreover, these terpenes were found in the xylem of healthy *P. massoniana* in different ratios, compared to the ratio of terpenes produced by larval *M. alternatus,* which might be involved in attraction of the insect vector. The basis for a chemoecological relationship between PWN and *M. alternatus* was suggested as based on these three volatile terpenes (Zhao *et al.*, 2007b).

The spread of pine wilt disease around the world has been caused by several aspects, i.e., climatic conditions, topographic and biological factors, as well as by humans, special timber exports (Futai, 2008).In Portugal, the nematode was detected for the first time in 1999 by Mota and coworkers in the Setúbal area (Mota *et al.*, 1999). The infection was controlled in this area by using a border belt area. However, those efforts were not enough and the PWD was spread to all the country. In 2008, it was detected in the district of Coimbra (Rodrigues *et al.*, 2009; Rodrigues, 2008) and in 2012 it was detected on Madeira island (Fonseca *et al.*, 2012). In 2008, the Portuguese Government unified different research institutes (research lines) inside Portugal in order to combine efforts to better understand the disease, control PWN-spread and eventually to find a cure ("Portaria n.° 553-B/2008," 2008).

On the other hand, bacteria have been founded as part of endophytic microbial community in *Pinus* spp. trees (Bal, 2003; Carrell & Frank, 2012; Izumi *et al.*, 2008; Pirttilä *et al.*, 2000, 2008; Proença *et al.*, 2011; Shishido *et al.*, 1995; Strzelczyk & Li, 2000) and it is likely that the endophytic bacteria might be carried by PWN into *Monochamus* spp. (Vicente *et al.*, 2013).

In the process of PWD, a role for symbiotic bacteria has been proposed , since several bacteria genera have been found associated with *B. xylophilus* (Han *et al.*, 2003; Higgins *et al.*, 1999; Proença *et al.*, 2010).

When colonizing the pine wood, the PWN will be in contact with the tree's endophytic microbial community. Despite its recognized importance, the number of endophytic microbial species known and described represents only a small fraction of the microbial diversity found in such habitats (Pace, 1997).

It is therefore necessary to assess and characterize the diversity of endophytic microbial community in pine trees to understand how they can play a role on PWD.

1.3. Plant-microbe interactions

1.3.1. Localization of bacterial communities in/on plants

The relationships between all kinds of life on Earth are complex and even though plants and microbes belong to three different biological domains - Bacteria, Archaea and Eucarya (Woese *et al.*, 1990) - there countless interactions of microbes with multicellular hosts including plants. Plants constitute a quite complex microbial ecosystem comprising different tissues or intercellular spaces spanning from the roots up to the shoots and leaves, all of with may be colonized by microbes (reviewed in Reinhold-Hurek & Hurek, 2011). Comparatively, the density of the microbial community at the rhizosphere is generally higher than the density of the endophytic microbial community that lives inside the plant (Compant *et al.*, 2010; Hallmann *et al.*, 1997). In addition, the plants feature distinct microbial communities depending on the plant species and these microbial communities may strongly interact with plants (Ma *et al.*, 2011; Rosenblueth & Martínez-Romero, 2006).

1.3.2. Mechanisms involved in plant-microbes interactions

Plants employ defense mechanisms to hinder the colonization of microbes and put a strain on the invaders for adaptation to the host. Such defenses comprise e.g., reactive oxygen species (ROS) or nitric oxide (NO), known as part of the innate immunity system of plants (Apel & Hirt, 2004; Zeidler *et al.*, 2004). Conversely, microbes counter plant defenses with antioxidant enzymes needed for plant colonization, such as superoxide dismutase (SOD), glutathione-reductase (GSR), catalase or laccase (Hartmann *et al.*, 2008).

Moreover, another example of a selection factor by plants is related to the use of certain substrates not readily usable by microorganisms and/or the use of specific physicochemical conditions such as high concentrations of sucrose in sugar cane (Oliveira *et al.*, 2009).

On the other hand, the role of some bacteria on plant growth is also recognized. Plant growth-promoting bacteria (PGPB) are found in soil, rhizosphere or inside the plant (Hardoim *et al.*, 2008; Ryan *et al.*, 2008). Some of beneficial effects from the

rhizosphere bacteria (rhizobacteria) and other PGPB to plant hosts are related to: i) production of phytohormones that support plant growth such as auxins, ethylene, gibberellins, cytokinins and abscisic acid (Gamalero & Glick, 2011); ii) production of enzymes, such as bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase, are involved in reduction of ethylene levels in plants; iii) phosphate and zinc solubilization, iron chelation by siderophores, nitrogen fixation and nitric oxide production; and iv) plant protection against pathogenic organisms by producing antagonistic compounds (such as antibiotics, osmolytes, organic acids and biosurfactants) or by inducing systemic resistance (ISR) of plants (Figure 1.3) (Gamalero & Glick, 2011; Glick *et al.*, 2007; Hardoim *et al.*, 2008; Reinhold-Hurek & Hurek, 2011; Ryan *et al.*, 2008; Stoltzfus *et al.*, 1997).

Furthermore, the importance of bacteria in legume nodulation is also well recognized. Here, the modulation of plant ethylene levels is also known as a function of bacteria, for example, by producing ACC deaminase which increases legumes nodulation by ACC (the immediate ethylene precursor) cleavage, to form ammonia and α-ketobutyrate, resulting in reduction of the ethylene concentration in the plant (Hardoim *et al.*, 2008; Rashid *et al.*, 2012). The ethylene is a substance involved in plant disease resistance, plant growth, interactions between plant and microbes (Glick *et al.*, 2007; Hardoim *et al.*, 2008).

Similarly, bacterial biosynthesis of indole-3-acetic acid (IAA), an example of an auxin, has been shown to promote and to benefic plant elongation and development (Lambrecht *et al.*, 2000). The cross-talk between ethylene and IAA is affected by bacterial ACC deaminase, i.e., the presence of ACC deaminase reduces ethylene levels and allows IAA biosynthesis with benefits on plant development. On the other hand, the high levels of ethylene inhibits the IAA biosynthesis (Glick *et al.*, 2007; Hardoim *et al.*, 2008; Lambrecht *et al.*, 2000).

Although, based on plant-bacteria interactions and their beneficial or harmful relationship, bacteria can be classified into endophytes or phytopathogens, respectively. This classification is not strict, for the ratio beneficial to pathogenic is dependent on factors such as environmental conditions or the balance with other bacterial populations (Andrews & Harris, 2000; Kloepper *et al.*, 1992; Sabaratnam & Beattie, 2003).

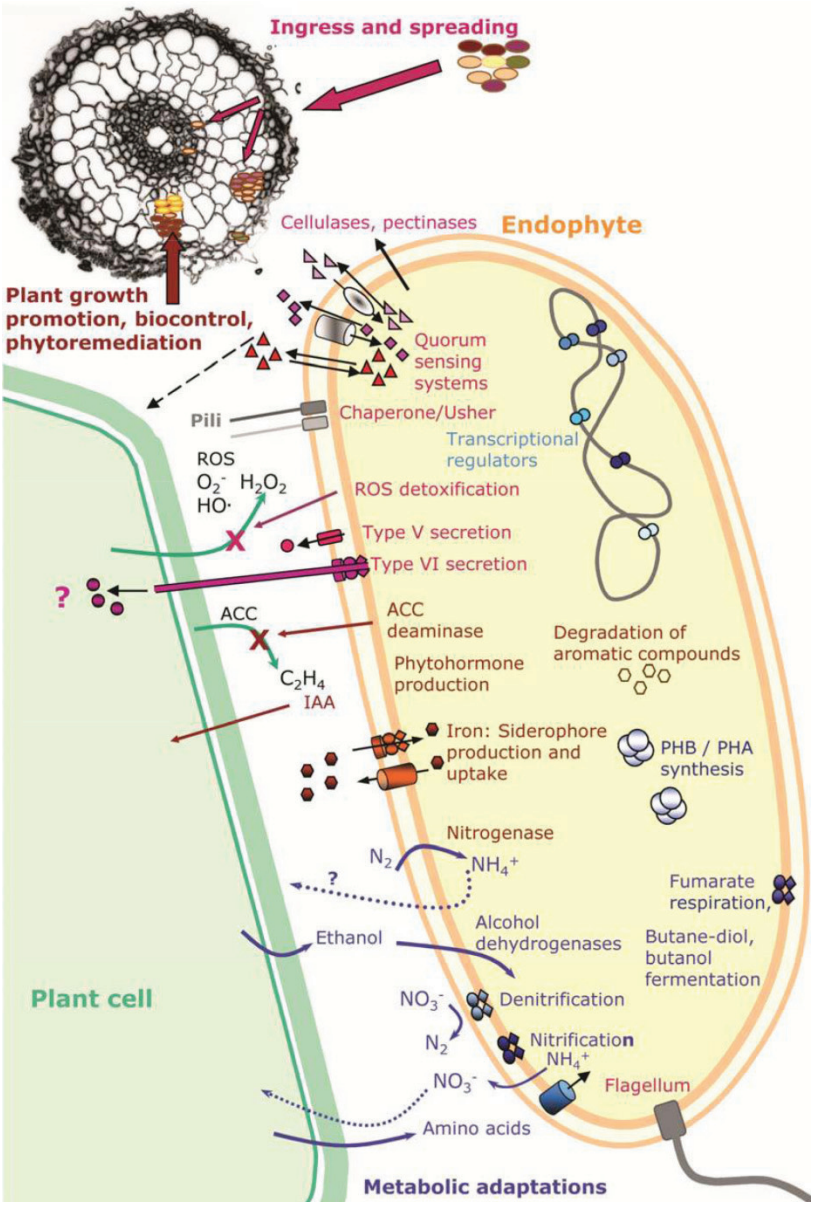


Figure 1.3. Interactions between endophytic bacteria and rice (*Oryza sativa*) cells based on analysis of the endophytic metagenome. Processes putatively related to: i) invasion, spread and establishment within the roots (red); ii) plant growth promotion, biocontrol, and phytoremediation (brown); metabolic adaptations (blue). The question mark (?) signifies unknown effector proteins produced and secreted and unknown transfer of ammonia (after nitrogen fixation) into the plant, respectively. Adapted from Sessitsch *et al.* (2012).

1.3.3. Endophytic bacteria

According to etymology, the term endophyte means inside the plant ("endo" means "inside" and "phyte" means "plant"). The term endophyte was previously defined as 'an organism inhabiting plant organs that at some time in its life can colonize internal plant tissue without causing apparent harm to the host' (Petrini, 1991). In addition,

endophytic bacteria have been defined as bacteria colonizing the internal plant tissues without causing harm to host and able to establish a mutualistic association (Hallmann *et al.*, 1997). Therefore, the endophytes were found to reside in the roots, stems and leaves of plants, and bacteria from more than one hundred genera have been identified in a broad range of plants, including woody plants and arable crops (Lodewyckx *et al.*, 2002; Rosenblueth & Martínez-Romero, 2006; Ryan *et al.*, 2008).

Bacterial endophytes may be classified as obligate or facultative endophytes. Obligate endophytes are strictly dependent on host plants for their survival, no life stages occur outside the plant except for plant-to-plant (vertically) or plant-to-insect-to-plant (by insect vectors) transmission to other host plants. On the other hand, facultative endophytes may live during their life cycle inside or outside plants (Hardoim *et al.*, 2008). For example, *Enterobacter cloacae* strain AS1 was identified as an obligate endophyte of pollen grains of Mediterranean Pines and to able to produce IAA (Madmony *et al.*, 2005).

Moreover, obligatory endophytic diazotrophs from gramineous plants were isolated including species of the genera *Azoarcus*, *Gluconacetobacter* and *Herbaspirillum* (Döbereiner *et al.*, 1993). In a recent study, researchers suggested the following species as obligate endophytes: *Mycobacterium* sp. T126, *Pedobacter terrae* strain QT16, *Pseudomonas* sp. Marine-13 and *Variovorax* sp. LZA10, since that these species were found in the roots from different sampling fields and but from the same stock of seeds (Croes *et al.*, 2013).

Conversely, facultative endophytes are more abundant than obligate endophytes and belong the *Proteobacteria* or *Actinobacteria*, but also to the phyla *Firmicutes* and *Bacteroidetes,* (Reinhold-Hurek & Hurek, 2011; Rosenblueth & Martínez-Romero, 2006).

Bacterial colonization of plant tissues can occur in different ways. Usually, some rhizosphere bacteria colonize plants through lateral root emergence, and then they colonize local root tissues, followed by migration throughout internal plant tissues from stem to leaves (Chi *et al.*, 2005). Another way of bacterial colonization of plant tissues occurs through wounds produced to the plant host by external agents, such as nematodes, insects or other mechanical damages (Linit, 1988). In addition, the active penetration of endophytic bacteria in plant tissues seems to be accomplished through the use of hydrolytic enzymes, such as cellulases and pectinases (Quadt-Hallmann *et al.*,

1997). Therefore, bacteria may be classified as "competent endophytes" or "opportunistic endophytes", depending on the endophyte possessing the key genetic machinery required for colonization or not (Hallmann *et al.*, 1997; Zinniel *et al.*, 2002).

1.3.4. Phytopathogenic bacteria

Phytopathogenic bacteria are able to cause harm and diseases to plant hosts. Some bacteria have been reclassified because of advances in taxonomy such as molecular polyphasic employing different, hierarchical approaches for better bacterial classification (Vandamme *et al.*, 1996). Currently, 39 genera are known to include phytopathogenic species: *Acetobacter, Acidovorax, Agrobacterium, Arthrobacter, Bacillus, Burkholderia, Brenneria, Clavibacter, Clostridium, Corynebacterium, Curtobacterium, Dickeya, Enterobacter, Erwinia, Ewingella, Gibbsiella, Gluconobacter, Herbaspirillum, Janthinobacterium, Leifsonia, Nocardia, Pantoea, Pectobacterium, Pseudomonas, Ralstonia, Rathayibacter, Rhizobacter, Rhizobium, Rhizomonas, Rhodococcus, Samsonia, Serratia, Sphingomonas, Spiroplasma, Streptomyces, Tatumella, Xanthomonas, Xylella* and *Xylophilus* (http://www.eppo.int/QUARANTINE/quarantine.htm; Bull *et al.*, 2010, 2012).

Moreover, a study by Mansfield and coworkers (2012) defined the top ten plant pathogenic bacteria according to scientific/economic importance: 1) *Pseudomonas syringae* pathovars; 2) *Ralstonia solanacearum*; 3) *Agrobacterium tumefaciens*; 4) *Xanthomonas oryzae* pv. *oryzae*; 5) *Xanthomonas campestris* pathovars; 6) *Xanthomonas axonopodis* pathovars; 7) *Erwinia amylovora*; 8) *Xylella fastidiosa*; 9) *Dickeya* (*dadantii* and *solani*); 10) *Pectobacterium carotovorum* (and *Pectobacterium atrosepticum*). In addition, "honorable" mentions were for *Clavibacter michiganensis* (*michiganensis* and *sepedonicus*), *Pseudomonas savastanoi* and Candidatus *Liberibacter asiaticus* (Mansfield *et al.*, 2012).

The ability to cause plant harm by bacterial pathogens in plants is due to: i) secretion of wall degradative enzymes, as cellulases and pectinases (Quadt-Hallmann *et al.*, 1997); ii) toxins production; iii) extracellular polysaccharide (EPS) production causing host xylem vessel blockage, for example by *Xylella fastidiosa* (Roper *et al.*, 2007); iv) excess of auxin levels (IAA) causing tumors; v) production of effector proteins that block the induced systemic resistance (ISR); vi) production of secretion systems induced by *hrp* (hypersensitive response and pathogenicity) or *avr* (avirulence) genes (Alfano & Collmer, 1996).

However, in forest trees, for example, pine trees, eucalyptus and corks, there are no reported diseases associated with the presence of phytopathogenic bacteria. In these trees various diseases are caused by fungi and other organisms, for example, insects or nematodes (Cooper & Gardener, 2006).

1.3.5. Endophytic bacteria in *Pinus* spp.

Studies of endophytic microbial diversity in wooden plants are less frequently conducted compared to that on seasonal ones (sugar cane, rice and bean, etc.). As one of the most abundant trees in many temperate forests, *Pinus* spp. constitute a vast habitat for bacteria. Thus, in recent years, studies aimed to investigate the endophytic microbial community of several pine trees species. The presence of endophytic bacteria has been reported e.g., in *P. contorta* (Bal *et al.*, 2012; Shishido *et al.*, 1995), *P. sylvestris* (Izumi *et al.*, 2008; Pirttilä *et al.*, 2000, 2008; Strzelczyk & Li, 2000) and *P. flexilis* (Carrell & Frank, 2012) (Table 1.1).The major bacterial genera in Scots pine, *P. sylvestris,* vary, according to different authors, but comprise e.g., *Methylobacterium* (Pirttilä *et al.*, 2000), *Pseudomonas* (Pirttilä *et al.*, 2000; Strzelczyk & Li, 2000), or *Bacillus* and *Paenibacillus* (Izumi *et al.*, 2008). The first study of endophytic bacteria in *Pinus* spp. reported the isolation of a siderophore-producing *Pseudomonas* from *P. sylvestris*, near the coastal Baltic Sea of Poland, that was identified based on the production of a yellow-green pigment (pyoverdine) on King's medium (Strzelczyk & Li, 2000). Moreover, the identification *M. extorquens* and *P. synxantha* isolated from buds of Scots pines in northern Finland, were based on 16S rRNA gene sequence as well as physiological characteristics and fatty acids profile (Pirttilä *et al.*, 2000). In a study performed by Izumi and coworkers (2008) in Scots pines from Scotland three isolates belonged to *Paenibacillus pabuli*, *P. wynii* and *Bacillus arvi* (Izumi *et al.*, 2008). Also, by performing Denaturing Gradient Gel Electrophoresis (DGGE) to evaluate the bacterial endophyte community, the authors concluded that the diversity of microbial communities is different between rhizosphere and root (Izumi *et al.*, 2008). Strains belonging to the genera *Paenibacillus* and *Bacillus* were also found as endophytes of Lodgepole pine, *P. contorta* (Bal, 2003; Bal *et al.*, 2012; Shishido *et al.*, 1995). According to Shishido and coworkers (1995), the strain isolated from Lodgepole pine near Williams Lake, B.C., Canada, within surface-sterilized root was identified as *B. polymyxa* according to the BIOLOG assay, however GC-FAME analysis was not conclusive

(Shishido *et al.*, 1995). According to Bal and coworkers (2012) (previous results published in (Bal, 2003)), the identification of bacterial strains was performed by GC-FAME and only for three strains the 16S rRNA gene was sequenced (Bal *et al.*, 2012). These strains were identified as *Paenibacillus polymyxa* (with same similarity to *P. peoriae*), *Dyadobacter fermentans* and were able to reduce acetylene (Bal *et al.*, 2012).

More recently, the major foliar endophytic bacterial community in the limber pine, *P. flexilis,* in Niwot Ridge, USA, was identified as belonging to the class of *Alphaproteobacteria* (50-70%), family *Acetobacteraceae*, and species of the genera *Gluconacetobacter* and *Acetobacter* (combined 45-60%) by using cultivation-independent methods (i.e., 454 pyrosequencing) (Carrell & Frank, 2012). Furthermore, nitrogen-fixing bacteria were found in foliar endophytic community by PCR amplification of the *nifH* gene (Carrell & Frank, 2012).

Different methodologies based on cultivation-dependent and -independent methods were performed in studies described above to better understand the endophytic microbial diversity. However, the overall differences in endophytic microbial community might also be due to different soil compositions and plant species in different sampling places, which is known to affect, for example, the pseudomonad-populations (Latour *et al.*, 1996).

The combination of culture-dependent methods and molecular methods allow a better understanding of endophytic microbial diversity.

Table 1.1. Bacterial endophytes identified in their respective *Pinus* host species

Endophyte genera	Endophyte Species	Host pine tree	Plant tissue	Reference
Acetobacter	*Acetobacter* sp.	*P. flexilis*	Needle	Carrell & Frank (2012)
Bacillus	*Bacillus* sp.	*P. contorta*	Needle	Bal *et al.* (2012)
			Stem	
			Roots	
	B. arvi	*P. sylvestris*	Roots	Izumi *et al.* (2008)
	B. licheniformis	*P. contorta*	Stem	Bal *et al.* (2012)
	B. longisporus		Stem	
	B. megaterium		Stem	
	B. mycoides		Stem	
	B. polymyxa		Roots	Shishido *et al.* (1995)
	B. pumilus		Needle	Bal *et al.* (2012)
			Stem	
			Roots	

(continued)

Table 1.1. (Continued)

Endophyte genera	Endophyte Species	Host pine tree	Plant tissue	Reference
Brevibacillus	*Brevibacillus* sp.		Stem	Bal *et al.* (2012)
Brevundimonas	*Brevundimonas vesicularis*		Stem	
Burkholderia	*Burkholderia pyrrocinia*		Stem	
Cellulomonas	*Cellulomonas biazotea*		Stem	
Dyadobacter	*Dyadobacter fermantans*		Stem	
Gluconacetobacter	*Gluconacetobacter* sp.	*P. flexilis*	Needle	Carrell & Frank (2012)
Kocuria	*Kocuria kristinae*	*P. contorta*	Stem	Bal *et al.* (2012)
	K. rosea		Stem	
Methylobacterium	*Methylobacterium extorquens*	*P. sylvestris*	Buds	Pirttilä *et al.* (2000)
Paenibacillus	*Paenibacillus* sp.	*P. contorta*	Needle	Bal *et al.* (2012)
			Stem	
	P. gordonae		Needle	
	P. pabuli		Needle	
		P. sylvestris	Roots	Izumi *et al.* (2008)
	P. peoriae	*P. contorta*	Stem	Bal *et al.* (2012)
	P. polymyxa		Needle	
			Stem	
	P. wynnii	*P. sylvestris*	Roots	Izumi *et al.* (2008)
Pseudomonas	*Pseudomonas sp.*	*P. contorta*	Stem	Bal *et al.* (2012)
		P. sylvestris	Roots	Strzelczyk & Li (2000)
	P. synxantha		Buds	Pirttilä et al. (2000)
Genera belong to *Alphaproteobacteria* (class)		*P. flexilis*	Needle	Carrell & Frank (2012)
Genera belong to *Acetobacteraceae* (family)		*P. flexilis*	Needle	Carrell & Frank (2012)

1.4. Nematode-bacteria interactions

Nematodes are one of the most abundant groups of animals, representing 80% of the total species-number, and are distributed in aquatic, air and terrestrial habitats (Holterman *et al.*, 2006; Lambshead & Boucher, 2003; Murfin *et al.*, 2012). Comprising total of 1 million nematode species are classified into 12 clades within the phylum Nematoda (Holterman *et al.*, 2006; Lambshead & Boucher, 2003). According to the food source, nematodes can be classified into groups of nematodes that are bacterial-, fungal- and plant-feeders, predators and omnivores.

Nematodes are involved in several humans diseases (e.g., lymphatic filariasis and onchocerciasis) and constitute important agriculture pests (e.g., affecting cereals, tomato, soybean, rice, pine trees) which cause a tremendous impact to economy (Murfin *et al.*, 2012; Turner & Rowe, 2006).

Nematode-bacteria interactions can be beneficial - mutualistic relationship, or harmful - parasitic/pathogenic relationship. As an example, the nematode *Caenorhabditis elegans*,

is a bacteria-feeding nematode, can be used as a model for human infectious diseases and permits to evaluate the pathogenicity of bacteria (Couillault & Ewbank, 2002; Irazoqui *et al.*, 2010; Pukkila-Worley & Ausubel, 2012; Waterfield *et al.*, 2008). *Bacillus nematocida* B16 is harmful to *C. elegans* by production of volatile organic compounds (VOC) attracting it and when inside the nematode intestine secrete two proteases that lead to nematode death (Niu *et al.*, 2010). Mutualistic relationships have been studied by entomopathogenic nematodes (EPN) and their bacterial associates (*Xenorhabdus* and *Photorhabdus*) (Murfin *et al.*, 2012; Ryss *et al.*, 2011).

Plant cell-wall-degrading enzymes have been demonstrated to be important in host-parasite interactions. *B. xylophilus* produces cellulases (β-1,4-endoglucanses) and pectate lyases that permit invasion of plant tissues (Kikuchi *et al.*, 2004, 2006, 2011). Because such proteins are secreted by bacteria and fungi, this suggests horizontal gene transfer (HGT) from bacteria and fungi to nematodes (Kikuchi *et al.*, 2011; Scholl *et al.*, 2003). These findings suggest a co-evolution process and stimulate the interest on the study of plant parasitic nematodes (PPN) interactions with bacteria (Bird & Koltai, 2000; Scholl & Bird, 2011).

1.4.1. Bacteria associated with Nematodes

Several studies reported bacterial symbionts associated with nematodes (Taylor *et al.*, 2012).

Mutualistic symbiosis have been studied between EPN of the genera *Heterorhabditis* or *Steinernema* and bacterial *Gammaproteobacteria* endossymbionts of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Forst & Nealson, 1996; Goodrich-Blair & Clarke, 2007; Murfin *et al.*, 2012; Ryss *et al.*, 2011). EPN were able to use bacterial symbionts as a food source or to use essential nutrients produced by bacteria that are essential for efficient nematode proliferation (Hu & Webster, 2000; Isaacson & Webster, 2002; Murfin *et al.*, 2012; Ryss *et al.*, 2011). Additionally, bacteria are also able to kill nematode host larvae and thus, EPN may be used as biological insect-control agents (Ryss *et al.*, 2011).

1.4.2. Bacterial diversity associated with *B. xylophilus*

In order to understand the role of bacteria in PWD it is necessary to know the diversity of the bacteria carried by PWN. Different bacterial genera have been isolated

from *B. xylophilus* from different pine hosts and also from different countries affected by PWD (Table 1.2).

The first report of bacteria associated with *B. xylophilus* by Oku and coworkers (1980) found that *Pseudomonas* was carried by PWN (Oku *et al.*, 1980), and other works followed reporting strains from the genus *Bacillus* (Kawazu *et al.*, 1996a) carried by PWN in Japan. Since then, many bacteria were observed by using electron microscopy attaching to the surface of PWN (Zhao *et al.*, 2000). PWN isolated from naturally infected *P. thunbergii* carry bacteria in average number of 290 per adult nematode surface (Guo *et al.*, 2002, 2007; Kusunoki, 1987; Zhao *et al.*, 2000). Moreover, bacteria from the genera *Xanthomonas* were also found associated with *B. xylophilus* (Higgins *et al.*, 1999). Researchers in China isolated *Pantoea*, *Peptostreptococcus*, *Enterobacter*, *Serratia*, *Staphylococcus*, *Buttiauxella*, *Stenotrophomonas* and the most frequently isolated genus was *Pseudomonas* (Han *et al.*, 2003; Tian *et al.*, 2010; Zhao & Lin, 2005; Zhao *et al.*, 2003). Moreover, the genera *Pantoea* and *Pseudomonas* were absent from trees not infected with *B. xylophilus* (Han *et al.*, 2003). Bacteria from the species *Burkholderia arboris*, *Brevibacterium frigoritolerans*, *Enterobacter asburiae*, *Ewingella americana* and *Serratia marcescens* were found in Republic of Korea associated with the nematode (Kwon *et al.*, 2010).

On the other hand, the first reports of the structural diversity of bacteria did not show the presence of bacteria inside *B. xylophilus*, contrary to what has been described for the EPN (Goodrich-Blair, 2007). Recently it was demonstrated that bacteria associated with PWN in Portugal were mainly *Pseudomonas*, *Burkholderia*, and *Enterobacteriaceae* (genera *Yersinia*, *Serratia*, *Ewingella*, *Pantoea* and *Erwinia*) (Proença *et al.*, 2010). Those results were also confirmed by a second study (Vicente *et al.*, 2011b). Bacteria belonged to the genus *Burkholderia* associated with PWN were for the first time reported in Portugal (Proença *et al.*, 2010). Although, *Burkholderia* spp. are usually part of endophytic microbial communities from different plants species (Suárez-Moreno *et al.*, 2012). Moreover, a study performed by Roriz and coworkers (2011) found the genera *Bacillus*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Paenibacillus*, *Pantoea* and *Terribacillus* associated with PWN (Roriz *et al.*, 2011). However, the PWN in this case were artificially introduced into pines and thus results obtained cannot be compared with *in* and *ex situ* studies (data are not included in Table 1.2).

Methodologies used to identify bacterial isolates in countries with PWD were very different. Some studies characterized isolates by culture techniques or based on identification by kits (Han *et al.*, 2003; Kawazu *et al.*, 1996a; Kwon *et al.*, 2010; Oku *et al.*, 1980; Roriz *et al.*, 2011; Tian *et al.*, 2010; Zhao & Lin, 2005; Zhao *et al.*, 2003) while others sequenced the 16S rRNA genes and compared them with international databases (Proença *et al.*, 2010; Roriz *et al.*, 2011; Vicente *et al.*, 2011b; Wu *et al.*, 2013; Zhu *et al.*, 2012).

It is likely that bacteria carried by PWN may play a role in pine wilt disease and some studies were aimed to understand their beneficial or harmful role on PWD.

Table 1.2. Bacterial genera associated with PWN from different countries and different host *Pinus* spp.

Country	Bacterial genera carried by PWN	Host pine species	References
Japan	*Bacillus*	*P. densiflora*	Kawazu *et al.* (1996a)
	Pseudomonas		Oku *et al.* (1980)
China	*Achromobacter*	*P. massoniana*	Wu *et al.* (2013)
	Stenotrophomonas		Wu *et al.* (2013)
	Achromobacter	*P. taiwanensis*	Wu *et al.* (2013)
	Ewingella		Wu *et al.* (2013)
	Achromobacter	*P. thunbergii*	Wu *et al.* (2013)
	Buttiauxella		Zhao *et al.* (2003)
	Enterobacter		Zhao & Lin (2005); Zhao *et al.* (2003)
	Ewingella		Wu *et al.* (2013)
	Leifsonia		Wu *et al.* (2013)
	Pantoea		Han *et al.* (2003); Zhao & Lin (2005); Zhao *et al.* (2003)
	Peptostreptococcus		Zhao & Lin (2005); Zhao *et al.* (2003)
	Pseudomonas		Han *et al.* (2003); Zhao & Lin (2005); Zhao *et al.* (2003); Zhu *et al.* (2012); Wu *et al.* (2013)
	Rhizobium		Zhu *et al.* (2012)
	Serratia		Zhao & Lin (2005); Zhao *et al.* (2003)
	Staphylococcus		Zhao & Lin (2005)
	Stenotrophomonas		Tian *et al.* (2010); Wu *et al.* (2013)
Korea	*Brevibacterium*	*P. densiflora*	Kwon *et al.* (2010)
	Burkholderia		Kwon *et al.* (2010)
	Enterobacter		Kwon *et al.* (2010)
	Ewingella		Kwon *et al.* (2010)
	Serratia		Kwon *et al.* (2010)

(continued)

Table 1.2. (Continued)

Country	Bacterial genera carried by PWN	Host pine species	References
Portugal	*Burkholderia*	*P. pinaster*	Proença *et al.* (2010); Vicente *et al.* (2011b)
	Cronobacter		Proença *et al.* (2010)
	Curtobacterium		Proença *et al.* (2010)
	Enterobacter		Vicente *et al.* (2011b)
	Erwinia		Proença *et al.* (2010); Vicente *et al.* (2011b)
	Ewingella		Proença *et al.* (2010)
	Hafnia		Proença *et al.* (2010)
	Janthinobacterium		Proença *et al.* (2010)
	Klebsiella		Proença *et al.* (2010)
	Luteibacter		Proença *et al.* (2010)
	Pantoea		Proença *et al.* (2010); Vicente *et al.* (2011b)
	Pectobacterium		Vicente *et al.* (2011b)
	Pseudomonas		Proença *et al.* (2010); Vicente *et al.* (2011b)
	Rahnella		Proença *et al.* (2010); Vicente *et al.* (2011b)
	Serratia		Proença *et al.* (2010); Vicente *et al.* (2011b)
	Yersinia		Proença *et al.* (2010)

1.4.3. Role of bacteria carried by PWN on PWD

In the past years, some researchers have suggested that, bacteria associated with PWN play a role in PWD (Guo *et al.*, 2007; Han *et al.*, 2003; Higgins *et al.*, 1999; Oku *et al.*, 1980; Proença *et al.*, 2010; Vicente *et al.*, 2012; Zhao & Lin, 2005; Zhao *et al.*, 2009). Results from different studies indicated that bacteria carried by the PWN are phytotoxin-producer and interact with the nematodes. If true, this might have resulted from a long-term co-evolution process between the nematodes and the bacteria (Paracer & Ahmadjian, 2000; Zhao & Lin, 2005). As a consequence, it is unsurprising that the genome sequencing of two bacterial strains of the genera *Serratia* and *Pseudomonas*, typically associated with *B. xylophilus* in Portugal, has shown these strains to contain a large number of genes likely involved in pathogenicity to nematodes (Proença *et al.*, 2012a, b).

1.4.3.1. Harmful role of bacteria carried by PWN on PWD

Several studies have supported the idea of bacterial roles in PWD, acting as phytopathogens for *Pinus* spp. The first reports supporting this idea were in Japan (Kawazu & Kaneko, 1997; Kawazu *et al.*, 1996b; Oku *et al.*, 1980). However, not a single study identified bacteria as the only agent responsible for PWD. Oku and

coworkers (1980) observed rapid wilting of pine trees and suggested that this was a consequence of the role of the nematode and the bacteria it transmitted. The authors found that bacteria transmitted by the nematode, belonging to the genus *Pseudomonas,* were involved in production of toxins (Oku *et al.*, 1980). However, the ability of bacteria to produce toxins was lost two months after isolation (Oku *et al.*, 1980) indicative for extrachromosomal coding of the respective toxin genes.

Phenylacetic acid (PA)-producing bacteria were suggested as pathogens and might be involved in the disease mechanism of PWD (Kawazu *et al.*, 1996b). In Japan, *Bacillus cereus* strain HY-3 carried by pinewood nematode was showed to produced PA (Kawazu *et al.*, 1996b). PA permits generation and accumulation of benzoic acid (BA) and its conjugates in suspension of *P. thunbergii* cultured cells as well as in three years old trees (Kawazu *et al.*, 1996b). On the other hand, aseptic pine seedlings infected only by bacteria or by aseptic *B. xylophilus* did not wilt (Kawazu & Kaneko, 1997).

Based on these studies, several studies focused on the pathogenic role of bacteria carried by the nematodes were performed.

According to Han and coworkers (2003), the inoculation of aseptic nematodes (*B. xylophilus* or *B. mucronatus*) did not cause browning or wilt of *P. thunbergii* callus or seedlings (Han *et al.*, 2003). However, the combination of aseptic nematodes with *Pseudomonas* strains (Njh, Njt) caused severe symptoms and also a browning when *Pseudomonas* filtered liquid was applied to the callus. These authors suggested that a co-infection of PWN and bacteria (or toxins produced by bacteria) play an active role on development of PWD (Han *et al.*, 2003).

In addition, Zhao and coworkers (2003) did not find bacteria in healthy pine trees but found bacteria carried by PWN from diseased trees (Zhao *et al.*, 2003). Twenty four bacterial strains were isolated and 17 of them were identified as phytotoxin-producing. The majority of phytotoxin-producing bacteria belonged to the genus *Pseudomonas* (Zhao *et al.*, 2003). These authors performed inoculations of black pine seedlings and pine trees in greenhouse and field inoculations and showed that the presence of both PWN and its associated phytotoxin-producing bacteria were necessary to cause disease (Zhao *et al.*, 2003). The number of PWN and bacteria were found to increase when black pine trees changed needles into yellow or brown and when the studied trees were completely wilted (Xie & Zhao, 2008; Xie *et al.*, 2004).

Other authors proposed a mutualistic symbiotic relationship between the PWN and its associated bacteria of the genus *Pseudomonas* (Zhao & Lin, 2005). In this study, PWN reproduction was promoted by 10 strains of the genus *Pseudomonas* (previously isolated as bacteria carried by PWN (Zhao *et al.*, 2003)), namely *P. fluorescens* (GcM5-1A, ZpB2-1A), *P. putida* (GcM6-2A, GcM6-1A, ZpB1-2A, GcM2-3A), *Pseudomonas* sp. (HeM-139A, HeM2A, HeM1A) and *P. cepacia* (GcM1-3A), but 19 strains showed an inhibitory effect on PWN reproduction. On the other hand, the presence of PWN promoted the multiplication of 18 of the 29 bacterial strains. Although, mutualistic bacteria were suggested to be necessary to promote PWD, they must be carried by PWN invading the host pine trees, i.e., without PWN the bacteria were not able to invade pine trees (Zhao & Lin, 2005).

The strain *P. fluorescens* GcM5-1A (Zhao *et al.*, 2003) was evaluated in terms of toxicity to suspension cells and *Pinus thunbergii* seedlings (Guo *et al.*, 2007). According to Guo *et al.* (2007), two cyclic dipeptides, cyclo(-Pro-Val-) and cyclo(-Pro-Tyr-), were secreted by strain GcM5-1A and showed toxic activity to both suspension cells and seedlings. The two cyclic dipeptides were identified by combination of structure-based approaches, i.e., MS, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY spectra (Guo *et al.*, 2007). Moreover, a 50 kDa protein was secreted by the same strain, purified from the culture and the N-terminal sequence was showed to be similar to flagellin (Guo *et al.*, 2008). Flagellin as well as the two cyclic dipeptides, showed toxicity to suspension cells and seedlings of *Pinus thunbergii* (Guo *et al.*, 2008). Lately, Zhang and coworkers (2012) showed that flagellin when added to calli of *P. thunbergii in vitro* could promote PWN proliferation and its associated bacteria. Although, inoculations on dead or alive calli of *P. thunbergii,* pretreated with flagellin, showed much higher population levels of PWN and *P. fluorescens* GcM5-1A on dead calli (Zhang *et al.*, 2012a). Moreover, the recombinant protein (83 % identity similar with hypothetical protein PFLU2919 from *P. fluorescens* SBW25) with or without the putative signal peptide of *P. fluorescens* strain GcM5-1A showed toxicity to both suspension cells and seedlings of *P. thunbergii* (Kong *et al.*, 2010).

Burkholderia arboris KRICT1 carried by PWN, produced pyochelin which was reported to be phytotoxic to the pine seedling and calli of *P. densiflora* (Le Dang *et al.*, 2011). Pyochelin was shown to have stronger phytotoxicity compared with PA produced by *Bacillus* spp. and might be involved in the PWD wilting process (Le Dang *et al.*,

2011).These results supported the hypothesis of the role of bacteria and its toxins in the development of PWD.

In a different study, axenic *B. mucronatus* (a non-pathogenic species) and axenic *B. xylophilus* did not cause wilt, but when in the presence of pathogenic bacteria, both species of *Bursaphelenchus* were able to induce PWD (Zhao *et al.*, 2009). Furthermore, Zhao and coworkers (2011) showed PWD to be caused by a combination of aseptic PWN with seven bacterial isolates identified from the genus *Bacillus* and *Stenotrophomonas* (Zhao *et al.*, 2011).

As pointed out by Jones and coworkers (2008) some of the above studies have limitations because the tests were mostly performed *in vitro,* and used calli, young trees or seedlings. Additionally, only a few strains of the microbial diversity carried by PWN were evaluated (Jones *et al.*, 2008). Wu and coworkers (2013) showed that some bacterial species and their carbon metabolism were related to virulence of *B. xylophilus*, i.e., bacteria with a higher carbon utilization were related with most virulent *B. xylophilus* (Wu *et al.*, 2013).

Recently, strains from *Serratia* sp., *Enterobacter* sp. and *Pantoea* sp. associated to PWN showed to be able to induce PWD symptoms in *P. pinaster* seedlings (Vicente *et al.*, 2012). In contrast, inoculations of aseptic microcuttings and seedlings of *P. densiflora* with aseptic PWN inoculated with *Rhizobium* sp. and *Pseudomonas* sp. did not cause wilt (Zhu *et al.*, 2012).

On the other hand, it was also suggested that bacteria were able to produce toxic compounds involved in nematotoxicity to PWN (Proença *et al.*, 2012a, b). The role of bacteria carried by PWN play on PWD thus remains controversial.

1.4.3.2. Beneficial role of bacteria carried by PWN on PWD

In the previous section, it was mentioned that some bacteria carried by PWN might be involved in the virulence of PWN but the bacteria-nematode relationship and the mechanism of PWD remains unclear.

Some studies recognized several secondary metabolites produced by endophytic bacteria to be involved in abroad spectrum of roles, for example, having biotechnological potential, producing antibiotics, acting as biological control agents against plant pathogens as well as promoting in plant growth (Qin *et al.*, 2011).

For the first time, in 2010, bacterial diversity associated with PWN from *P. pinaster* was reported in Portugal (Proença *et al.*, 2010). As mentioned above in section 1.4.2, the isolates were identified and all belonged to the Gram-negative *Beta* and *Gammaproteobacteria*, except for one Gram-positive strain that belonged to the class *Actinobacteria*. The major bacterial populations associated with the nematodes were different depending of the sampled forest area, and none of the isolated bacterial species was found in nematodes from all forest areas tested. Moreover, 60 to 100% of the isolates, depending on sampled area, produced siderophores and at least 40% produced lipases. These bacteria were suggested to play a role in physiological plant response (Proença *et al.*, 2010). Siderophores, as iron biochelators, could be involved in potential plant growth promotion and plant protection but, on the other hand, bacterial siderophores were suggested inhibiting iron uptake by plants (Gamalero & Glick, 2011).

The ability of bacteria to produce lipases can be related to a beneficial role of bacteria protecting trees from PWD, because plant lipases as well as their hydrolysis products have been described as playing part in plant defense (monolaurate and oleic acids) (Hunzicker, 2009; Kwon *et al.*, 2009; Raffaele *et al.*, 2009). According to Proença and coworkers (2010) a few bacterial strains were able to produce proteases (Proença *et al.*, 2010).

More recently, two bacterial genomes of nematotoxic *Pseudomonas* sp. M47T1 and *Serratia* sp. M24T3 were sequenced (Proença *et al.*, 2012a, b). Both draft genomes showed multiple genes potentially involved in virulence and nematotoxic activity, such as the genes coding for colicin V and bacteriocin biosynthesis (Proença *et al.*, 2012a, b). The genome of these bacteria also include a set of genes typical for plant niche adaptation such as acetoin (diacetyl) reductase that could be involved in plant protection against fungal and bacterial infections (Proença *et al.*, 2012a, b). On the other hand, the genus *Serratia* was already reported as pathogenic to the pine sawyer beetle *Monochamus* (Shimazu, 2009). Therefore, some chemical compounds and other bio-products produced by bacteria carried by nematode have been suggested to control PWD.

1.5. Products active against PWNs

1.5.1. Microbial proteases

In order to find a strategy for control of PWD, several studies developed bioassays against the nematode *B. xylophilus* using proteases, essential plant oils or chemical products. Proteases represent a class of enzymes, found in plants, animals and microorganisms, and may be divided into two groups: i) intracellular proteases, with physiological roles inside the organisms, for example altering protein turnover, sporulation and conidial discharge, germination, enzyme modifications, nutrition, regulation of gene expression; ii) extracellular proteases, modifying protein catabolism roles outside the microorganisms, and may also act as an exotoxin causing consequent pathophysiological processes (Rao *et al.*, 1998). Proteases are also divided into exo- and endopeptidases based on their mechanistic action near the ends (N and C termini) or at the peptide bonds in the inner regions of the target polypeptide chain, respectively. Depending on the nature of the functional group at the active site, proteases are classified as aspartic proteases, cysteine proteases, metalloproteases and serine proteases. Proteases have a large variety of applications and are used for commercial proposes (Rao *et al.*, 1998).

Such hydrolytic enzymes produced by Gram positive bacteria have been identified to be involved in the degradation of nematode components (Cox *et al.*, 1981; Decraemer *et al.*, 2003; Huang *et al.*, 2005). Alkaline serine proteases have been reported to be produced by *Brevibacillus laterosporus* strain G4 (30 kDa designated BLG4) and from *Bacillus nematocida* (28 kDa) (Huang *et al.*, 2005; Qiuhong *et al.*, 2006). Although, both proteases were not the only virulence factor responsible for the nematicidal activities in these bacteria. According to Lian and coworkers (2007), an extracellular cuticle-degrading protease from *Bacillus* sp. strain RH219 designed Apr219 was reported as an important nematicidal factor, killing the nematodes in 12 h (Lian *et al.*, 2007).

A study described the Gram negative bacterium isolated from soil, *Stenotrophomonas maltophilia* G2, to have nematotoxic activity against *B. xylophilus*, killing 65% of the nematodes after 24 h of incubation (Huang *et al.*, 2009). The analysis of the virulence factors revealed the presence of an extracellular serine protease of 28 kDa able to digest the nematodes cuticle (Huang *et al.*, 2009). Among the hydrolytic enzymes, serine proteases have recently been shown to be very important in the penetration

and digestion of nematodes by nematode-trapping fungi (Ahman *et al.*, 2002; Lopez-Llorca *et al.*, 2002; Wang *et al.*, 2006; Yang *et al.*, 2005).

The production of serralysin-like proteases was detected in several *Serratia* strains related to insects (Kim *et al.*, 2007). A metalloproteinase is also produced by the entomopathogenic bacteria from the genera *Xenorhabdus* and *Photorhabdus* (*P. luminescens*), both symbiotic with their respective nematodes (Massaoud *et al.*, 2010).

1.5.2. Essential plant oils

Since time immemorial plants are recognized to produce extracts that may be used as drugs for different applications. Several studies reported essential plant oils, extracted from different plants, to have nematotoxic activity against *B. xylophilus* (Alen *et al.*, 2000; Barbosa *et al.*, 2010, 2012; Choi *et al.*, 2007; Elbadri *et al.*, 2008; Hong *et al.*, 2007; Kim *et al.*, 2008, 2011; Kong *et al.*, 2006, 2007; Park *et al.*, 2005, 2007). The essential oils of thyme, extracted from *Thymus vulgaris* (family *Lamiaceae*) (Kong *et al.*, 2006, 2007), and *T. caespititius* and *T. capitata* (Barbosa *et al.*, 2010, 2012), were found to be nematicidal against PWN with 100 % mortality. However, the most dominant component in oils was different depending on the plant species, i.e., thymol from *T. vulgaris* and carvacrol from *T. caespititius* and *T. capitata* (Barbosa *et al.*, 2010; Kong *et al.*, 2007). Besides the differences between the plant species, other essential oils and their compounds from *Cinnamomum verum*, *Eugenia caryophyllata* and *Mentha spicata* (Park *et al.*, 2005); *Trachyspermum ammi*, *Pimenta dioica* and *Litsea cubeba* (Park *et al.*, 2007); *Allium cepa* (Choi *et al.*, 2007); *Coriandrum sativum*, *Liquidambar orientalis* and *Valeriana wallichii* (Kim *et al.*, 2008); *Syzygium aromaticum*, *T. vulgaris* and *Pelargonium inquinans* (Elbadri *et al.*, 2008); *Chamaespartium tridentatum* (Barbosa *et al.*, 2010); *Origanum vulgare* and *Satureja montana* (Barbosa *et al.*, 2010, 2012) also showed effective nematicidal activity (100 %). Thus, essential oils constitute a bionematicidal tool against PWN beyond chemical pesticides.

1.5.3. Chemical compounds

Oh and coworkers (2009) screened 206 compounds with recognized activities against fungi and nematodes. The chemical compound HWY-4213 (1-*n*-undecyl-2-[2-fluorphenyl] methyl-3,4-dihydro-6,7-dimethoxy-isoquinolinium chloride),

$C_{29}H_{41}ClFNO_2$, a highly water-soluble protoberberine derivative, was identified as a dual-acting anti-nematicidal as well as an antifungal compound (Oh *et al.*, 2009). HWY-4213 was tested as nematicidal on 4 year-old *P. densiflora* seedlings injected two weeks before the infection with PWN strain YB-1 isolates, and showed a potent therapeutic nematotoxic activity (Oh *et al.*, 2009).

Moreover, emamectin benzoate, dissolved in water in order to reduce its phytotoxicity, showed its preventative effect against the wilt of PWN infected black pine trees, *P. thunbergii* (Takai *et al.*, 2001a).

Kwon and coworkers (2010) showed that oxolinic acid, an antibacterial agent, was effective against five of six bacteria carried by PWN and suppressed the development of PWD in three-year-old *P. densiflora* seedlings. It showed effectivity for PWD-control of 71% at a dosage of 3 mg per seedling. The best result for PWD prevention was obtained when oxolinic acid was mixed with abamectin, the nematicidal agent, compared to both compounds used alone (Kwon *et al.*, 2010).

1.6. Taxonomy in the era of sequencing

The previous sections introduced some definitions and interactions between bacteria, pine trees and nematodes. Herein, bacteria were recognized to play a significant role in different environments and could be used for different purposes.

Taxonomy is traditionally divided into classification, nomenclature and identification of new organisms (Kämpfer & Glaeser, 2012). All the prokaryote taxonomy is controlled by the Bacteriological Code (Lapage *et al.*, 1992). Prokaryote characterization is comprised of phenotypic and genetic methods (Tindall *et al.*, 2010; Vandamme *et al.*, 1996). A remarkable finding was the 16S rRNA gene that allowed, since then, establishing a taxonomy system based on molecular genetic relatedness, and made possible to relate the organisms phylogenetically (Fox *et al.*, 1977; Kämpfer & Glaeser, 2012; Rosselló-Móra, 2012; Tindall *et al.*, 2010; Vandamme *et al.*, 1996; Woese, 1987).

Definition and species concepts were changed over the years (Kämpfer & Glaeser, 2012; Rosselló-Móra, 2012). Previously, species as the basic unit of taxonomy was defined as "the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔTm. Both values must be considered" (Wayne *et al.*, 1987). Therefore, the phenotypic data

should be supported by the genomic information (Murray *et al.*, 1990; Tindall *et al.*, 2010; Wayne *et al.*, 1987).

The concept of polyphasic characterization was first introduced by Colwell (1970) and integrates genotypic and phenotypic information (Colwell, 1970). Later, the concept was improved by Vandamme and coworkers (1996) in a way "that would be consistent with phylogenetic classification" (Vandamme *et al.*, 1996). The phylogenies were based primarily on 16S rRNA or 23S rRNA genes sequences analyses (Vandamme *et al.*, 1996). Moreover, DNA-fingerprinting techniques were added that allowed and contributed for rapid and better differentiation, e.g. AFLP (amplified fragment-length polymorphism PCR), REP-PCR (repetitive extragenic palindromic-PCR), BOX-PCR (derived from the *boxA* element), ERIC-PCR (enterobacterial repetitive intergenic consensus sequences-PCR), RAPD (randomly amplified polymorphic DNA); RFLP (restriction fragment length polymorphism), ARDRA (amplified ribosomal DNA restriction analysis), PFGE (pulsed-field gel electrophoresis) (Figure 1.4) (Kämpfer & Glaeser, 2012; Rosselló-Móra, 2012; Tindall *et al.*, 2010; Vandamme *et al.*, 1996).

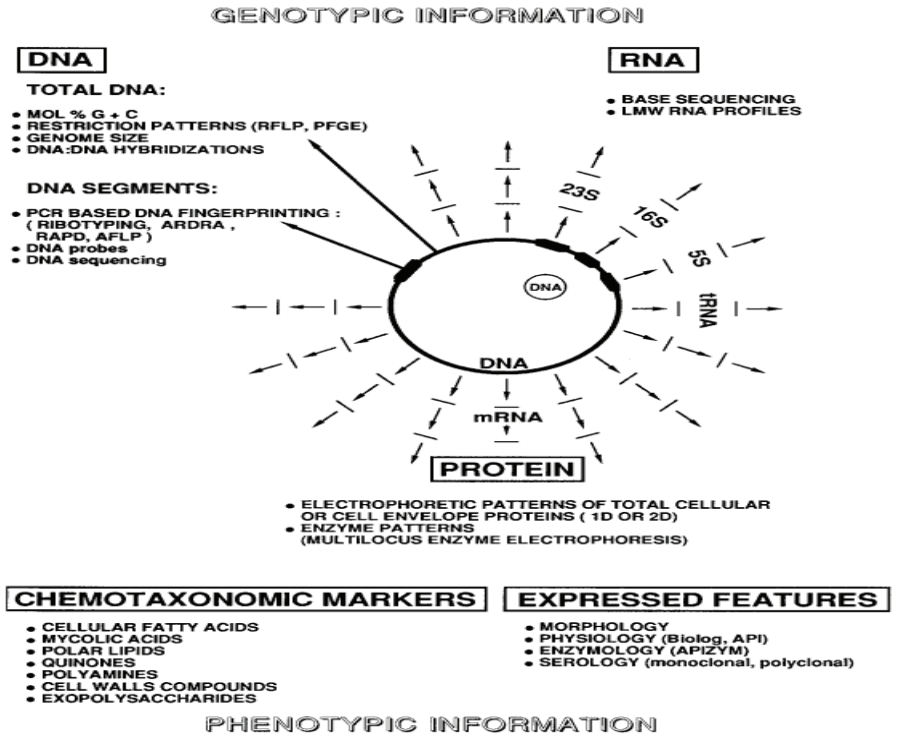


Figure 1.4. Schematic overview of genotypic and phenotypic information - diverse cellular components and analysis techniques used. RFLP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; ARDRA, amplified rDNA restriction analysis; RAPD, randomly amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; LMW, low molecular weight; 1D, 2D, one- and two-dimensional, respectively. Adapted from Vandamme *et al.* (1996).

However, all these fingerprinting techniques have some limitations. Moreover, for chemotaxonomic analysis it was necessary include fatty acids, polar lipids, respiratory quinones, pigments, polyamines, cell peptidoglycan, the presence or absence of teichoic and/or mycolic acids (Tindall et al., 2010). Therefore, Tindall and coworkers (2010) published full guidelines for species description in "Notes on characterization of prokaryote strains for taxonomic purposes" (Tindall et al., 2010). It is important to have a consensus from biochemical, physiochemical, serological, pathological, phylogenetic and genotypic properties for species definition.

In the genome sequencing era, new sequencing methodologies became more efficient and enable to analyze multiple genes and even whole genomes more quickly and at a higher quantity and quality.

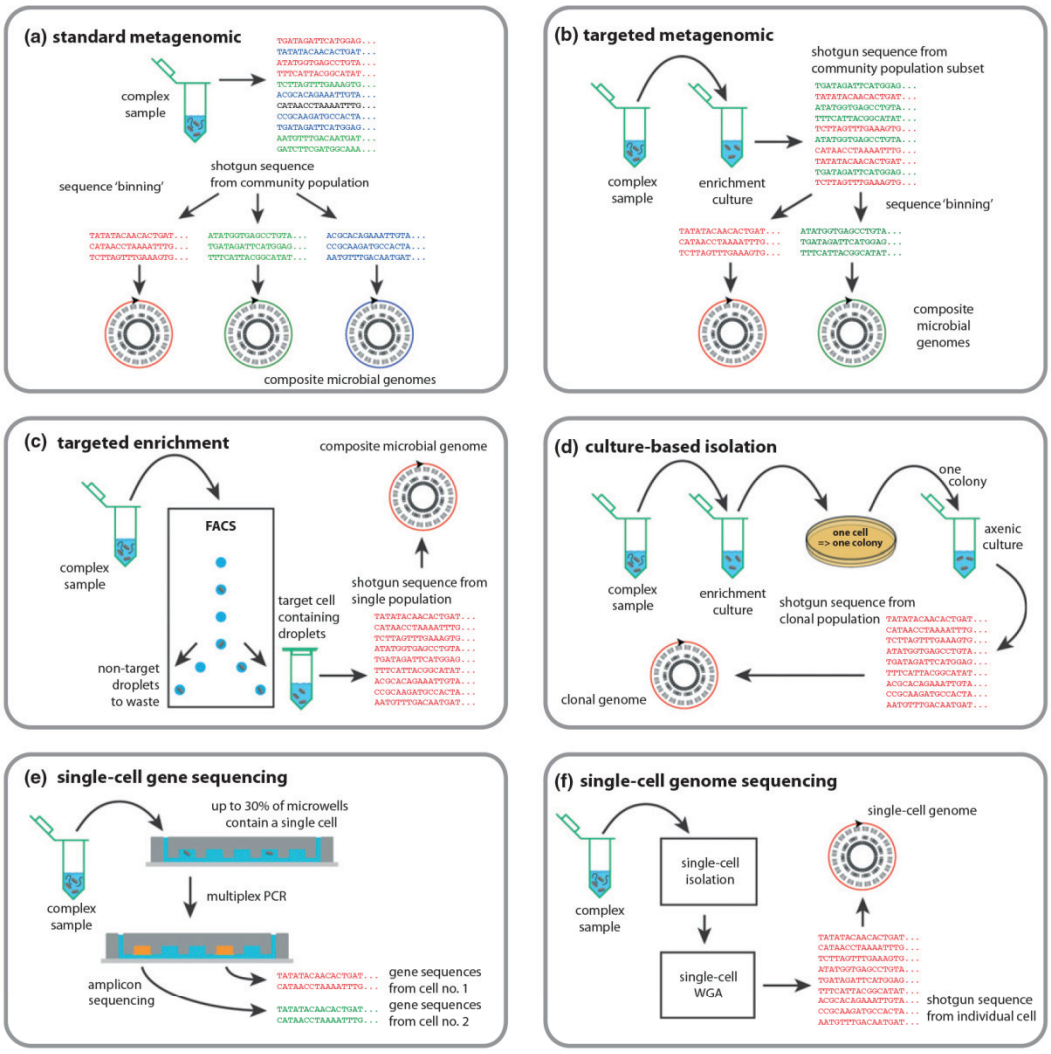
1.7. Bacterial genomics

At present, approximately 12,000 prokaryotic species have been valid taxonomically named. This number is very likely below that what can be expected living in different ecological niches, such as soil, water, plants, animals, etc.

The next-generation sequencing (NGS) has changed the way of sequencing and inflated the number of genomes available as the effective cost per base/genome has tremendously decreased since 2008 (http://www.genome.gov/sequencingcosts/). The genome sequence-based studies could overcome challenges related to i) species classification, ii) physiological and biochemical characteristics of bacteria as well as interaction mechanisms between organisms and iii) full genome analyzes potentially leading to new discoveries for biotechnological applications.

Different genome sequencing methods are used depending on the aims of the planned work, from single-cell genomics to metagenome sequencing, i.e. multiple genomes from various organisms in certain environment (Figure 1.5).

At the time of writing this, 12,867 genomes were completed, 26,300 genomes were incompleted and 961 genomes are in-progress (http://www.genomesonline.org/). From this number, only 2,312 genomes of the type strains are sequenced (http://ezgenome.ezbiocloud.net/?cm=ezgenome_chart) that representing only ~19 % of total type strains with a valid name.



(g) genome sequencing approaches

sample preparation approach	method-specific requirements					
	lab culture	read 'binning'	cell isolation	amplification	enabling technology	data type
standard metagenomic	no	yes	no	no	informatics	composite genome
targeted metagenomic	yes, enrichment	yes	no	no	informatics	composite genome
targeted enrichment	no	no	yes	often WGA	flow cytometry	composite genome
culture-based isolation	yes, axenic	no	often yes	no	traditional method	clonal genome
single-cell (gene sequencing)	no	no	yes	yes, PCR	cell isolation	linked gene sequences
single-cell (genome sequencing)	no	no	yes	yes, WGA	contamination-suppression	single-cell genome

Figure 1.5. Diversity of approaches for microbial genomic sequencing. Depending on the methodology choses the sequencing outcome can be very different, for example approaches a, c, e, f do not require a pure culture of the target organism(s). The final results of approaches a and b are multiple genomes whereas c, d and f typically lead to genomes of a single species. As a consequence approaches a and b rely heavily on bioinformatics methods such as binning. (g) Table summarizes key characteristics of the genomic methods depicted in parts (a–f). Adapted from Blainey (2013).

Until now, 19 endophytic bacterial genomes were completely sequenced belonging to the Classes of *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria* and *Gammaproteobacteria*, isolated from different environments summarized in Table 1.3.

Table 1.3. Endophytic bacterial strains from different hosts with completely sequenced genomes

Endophytic class	Endophytic strain	Isolation source	References
Actinobacteria	*Microbacterium testaceum StLB037*	Potato leaves	Morohoshi *et al.* (2011)
Alphaproteobacteria	*Gluconacetobacter diazotrophicus* Pal5	Sugarcane root	Bertalan *et al.* (2009)
	Azospirillum sp. B510	Rice stem	Kaneko *et al.* (2010)
Bacilli	*Bacillus subtilis* BSn5	*Amorphophallus konjac* calli tissue	Deng *et al.* (2011)
Betaproteobacteria	*Azoarcus* sp. BH72	Kallar grass roots	Krause *et al.* (2006)
	Burkholderia phytofirmans PsJN	Onion root	Weilharter *et al.* (2011)
	Burkholderia sp. strain KJ006	Rice root	Kwak *et al.* (2012)
	Variovorax paradoxus S110	Potato plant	Han *et al.* (2011)
Gammaproteobacteria	*Klebsiella pneumoniae* 342	Maize stem	Fouts *et al.* (2008)
	Pseudomonas putida W619	Poplar stem and root	Taghavi *et al.* (2009)
	Serratia proteamaculans 568	Poplar root	Taghavi *et al.* (2009)
	Stenotrophomonas maltophilia R551-3	Poplar stem, root and rhizosphere	Taghavi *et al.* (2009)
	Enterobacter sp. strain 638,	Poplar stem	Taghavi *et al.* (2009, 2010)
	S. plymuthica strain AS9	Rapeseed roots	Neupane *et al.* (2012a)
	S. plymuthica strain AS12	Rapeseed roots	Neupane *et al.* (2012b)
	S. plymuthica strain AS13	Rapeseed roots	Neupane *et al.* (2012c)
	Enterobacter cloacae subsp. *cloacae* strain ENHKU01	Pepper	Liu *et al.* (2012)
	Pseudomonas poae RE*1-1-14	Endorhiza of the sugar beet	Müller *et al.* (2013)
	Enterobacter sp. strain R4-368	Roots of *Jatropha curcas* L.	Madhaiyan *et al.* (2013)

The resulting endophytic genomes were chosen in order to understand the beneficial roles on plant growth-promoting by identification of those genes and also to discover new strategies for agriculture biotechnology. Moreover, the strains reported have been demonstrated, for example, a protective role against fungal and bacterial phytopathogens (*Xanthomonas oryzae* and *Xanthomonas albilineans*) (Bertalan *et al.*, 2009; Kaneko *et al.*, 2010). The genome information, size and structure seem to be related with bacterial life style resulting in diverse bacterial functions (Frank, 2011).

Moreover, comparison of bacterial endophytic genomes with bacterial genomes from different environments (for example clinical, soil, etc.) will allow a better understand of colonization and other mechanisms. The bacterium *S. maltophilia* was recognized as involved in pathogenesis in humans, as a nosocomial multidrug-resistant microorganism as well as pathogen of the *Xanthomonadaceae*. The complete genome of a nosocomial strain *S. maltophilia* K279a was sequenced (Crossman *et al.*, 2008). Moreover, the strain *S. maltophilia* G2, isolated from soil, was able to kill nematodes by produced extracellular serine protease (Huang *et al.*, 2009). Future genomic analysis will allow a better understanding of conserved genes of the species from different environments as well as the genes responsible for pathogenicity processes. Possibly, the comparison between draft and completed genomes will elucidate the differences between the genomes that affect the diversity of roles that can be assumed by the same bacterial species. In case of *Serratia marcescens*, this reaches from plant grow promoting to phytopathogen through the acquisition of genetic mobile elements or environmental stimuli (Ovcharenko *et al.*, 2010; Zhang *et al.*, 2005).

Cheng and coworkers (2013) by using metagenomic analysis of the PWN microbiome showed a symbiotic relationship between PWN and its microbiome for xenobiotics degradation (Cheng *et al.*, 2013). In this study, the major bacterial community belonged to the phylum *Proteobacteria* (95 %), including *Alphaproteobacteria* (46.6 %), *Gammaproteobacteria* (38.3 %) and *Betaproteobacteria* (10.1 %). *Bacteroidetes* (4.4 %) and other groups (0.6 %) were also assessed. Moreover, it was reported the presence of enzymes involved in the metabolism of xenobiotics (degradation of α-pinene) in genome of *Serratia* sp. M24T3 (Cheng *et al.*, 2013). *Serratia* sp. M24T3 was previously isolated from associated with PWN (Proença *et al.*, 2010) and its genome was published more recently (Proença *et al.*, 2012a).

The advances in molecular biology by new methodologies will provide a better knowledge of all the factors involved, such as, in pathways of production of nematotoxins as well as the bacterial-nematode-plant interactions. Furthermore, based on bacterial genomes sequencing and the correct application of this knowledge, the discovery of new products, for example microbial toxins or enzymes, could promote and formulate commercial nematicidal agents (Tian *et al.*, 2007).

1.8. Objectives

Emerging diseases of trees such as Pine Wilt Disease (PWD) pose a serious biological and financial threat to temperate forestry. The interplay between pathogenic nematode *B. xylophilus*, pine-plant and their microbiota for disease-progression is not well understood. This work aims to deciphering the composition and to understand the role of the microbial community within the host-plant upon nematode infection during PWD. The positive or negative contributions of endophytic, symbiotic bacteria to nematode-infection are the focus of this study and the specific goals are:

1. To assess the microbial endophytic community-structure of the host *Pinus pinaster* under different environments (different soils) and different physiological conditions (symptomatic versus non-symptomatic) and comparing it with the endophytic microbial community of other tree-species from the same environments;

2. To assess the microbial community associated with *B. xylophilus* during tree infection from Portugal and from the United States of America;

3. To characterize bacterial isolates from *P. pinaster* trees and, if possible, to describe new species;

4. To find bacterial metabolites produced by nematode-associated bacteria and to evaluate the nematotoxic properties of these compounds;

5. To sequence the genomes of bacteria producing nematotoxic compounds to identify genes involved;

6. To evaluate the ability of previously genome sequenced nematode-associated bacterial strains to work as a plant growth-promoting bacteria, using the plant model *Arabidopsis thaliana*.

2

Structural and Functional Microbial Diversity of the Endophytic Community in Pine Trees Affected to Pine Wilt Disease

Results in preparation for submission:
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2.1. Abstract

Pine wilt disease (PWD), caused by the pinewood nematode *Bursaphelenchus xylophilus*, is one of the most devastating forest diseases in the world and has created a tremendous economic, social and environmental impact. Portuguese pine forest is mainly comprised of PWD-susceptible *Pinus pinaster* trees. This study aimed to access the endophytic microbial (bacterial and archaeal) community of *P. pinaster* by molecular and cultivation methods, and to assess the diversity of genes responsible for endophytic functions such as those involved in nitrogen cycle, nitrogen fixation (*nifH* and *nifJ* genes) and nitric oxide production (*nirK* and *nirS* genes). Members of the classes *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria*, *Flavobacteria*, *Gammaproteobacteria* and *Sphingobacteriia* were identified from the sampling areas. *Gammaproteobacteria* were the most abundant bacteria. In addition, the members of classes *Bacteroidia*, *Deinococci*, *Fusobacteria, Spirochaetes, Planctomycetaceae* and *Verrumicrobiae* were only detected by DGGE. Furthermore, *Archaea* were found as part of the endophytic community, belonging to the phyla *Euryarchaeota, Thaumarchaeota* and *Crenarchaeota*. The diversity of *nifH* and *nifJ* genes suggested that the population of nitrogen fixing bacteria was diverse within the *Gamma-* and the *Alphaproteobacteria*. The prevalence of *nir* genes appeared to be more related with the sampling area and the structural diversity of the endophytic community (only *nirK* in Avô, mostly *nirS* in Mallhada) than with the physiological status of the trees. This study contributes to increase the knowledge about the endophytic microbial community of pine trees affected by PWD, focusing on Bacteria and Archaea and whether they have a role for the plant.

2.2. Introduction

Native to North America (the USA and Canada), pine wilt disease (PWD) is one of the most devastating forest diseases in the world. The disease has spread to Asia and to Europe, mainly affecting Japan, China, Korea, Taiwan, Portugal and Spain (EPPO/OEPP, 2009). Appearance of the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Nickle *et al.*, 1981), in these countries has created a tremendous economic, social and environmental impact (Tóth, 2011). *B. xylophilus* has been considered for a long time to be the only known causal agent of disease. Recently, a relationship of mutuality has been suggested between bacteria and PWN (Zhao & Lin, 2005). In Portugal, the first report of PWD was in 1999 by Mota and coworkers (Mota *et al.*, 1999), south of the Tagus river, and after 2008, the PWD had spread to the center and north part of the country (Rodrigues, 2008). Portuguese forests are mainly composed of species of pine, eucalyptus and oak trees. In Portuguese pines forests, the most abundant species, *P. pinaster*, making up 62.5 % of total trees, is PWD-susceptible tree (Rodrigues, 2008).

Several studies focused on the bacterial communities associated to PWN in order to determine their role in PWD (Chapter 4; Guo *et al.*, 2007; Han *et al.*, 2003; Higgins *et al.*, 1999; Proença *et al.*, 2010; Zhao *et al.*, 2009). Nevertheless, the diversity of the bacteria associated with PWN were shown to be different in each sampled country. In Japan, strains from the genera *Bacillus* and *Pseudomonas* were associated with PWN (Kawazu *et al.*, 1996a; Oku *et al.*, 1980). On the other hand, researchers in China isolated strains of different genera but the majority was *Pseudomonas* (Han *et al.*, 2003; Tian *et al.*, 2010; Zhao & Lin, 2005; Zhao *et al.*, 2003). In the Republic of Korea, strains of the genera *Burkholderia*, *Brevibacterium*, *Enterobacter*, *Serratia* and *Ewingella* were found (Kwon *et al.*, 2010). Recently, in Portugal, the bacteria associated with PWN were assessed and were mainly *Pseudomonas*, *Burkholderia*, and *Enterobacteriaceae* (Chapter 4; Proença *et al.*, 2010; Vicente *et al.*, 2011b).

Plants are one of the most interesting ecological niches for microorganisms and some studies showed the presence of an endophytic community in *Pinus* spp. trees (Reinhold-Hurek & Hurek, 2011; Ryan *et al.*, 2008). Therefore, the bacteria carried by PWN could be part of the endophytic microbial community. The presence of endophytes in plant tissues has been recognized for a long time to be positive for the plant. Thus, the term "endophytic bacteria" has been defined as bacteria able to colonize plant internal tissues without causing harm to the host and to be able to

establish a mutualistic association (Hallmann *et al.*, 1997). Endophytes are known to reside in roots, stems and leaves of plants, and bacteria of more than one hundred different genera have been found in a broad range of plants, including both woody and herbaceous plants used in agriculture (Lodewyckx *et al.*, 2002; Ryan *et al.*, 2008).

In Scots pine, *P. sylvestris*, the major genera of endophytic bacteria found were *Methylobacterium, Pseudomonas* (Pirttilä *et al.*, 2000, 2008; Strzelczyk & Li, 2000), *Bacillus* and *Paenibacillus* (Izumi *et al.*, 2008). *Paenibacillus* and *Bacillus* were also found as endophytes of *P. contorta* (Bal, 2003; Shishido *et al.*, 1995), while the genera *Acetobacter* and *Gluconacetobacter* were part of the foliar endophytic community of *P. flexilis* (Carrell & Frank, 2012).

Recently, Archaea were found to be part of the endophytic community of rice roots and coffee cherries (Oliveira *et al.*, 2013; Sun *et al.*, 2008). In regard to pine trees, no archaeal endophytes were reported , and, to the best of our knowledge, the closest information mentionsing archaea as part of the soil community in Pine Barrens Forest (NY, USA) (Shah *et al.*, 2011).

Endophytic bacteria have been shown to exhibit strong anti-fungal activity, antagonise bacterial pathogens and control plant parasitic nematodes (Brooks, D. S. *et al.*, 1994; van Buren *et al.*, 1993; Hinton & Bacon, 1995; Mukhopadhyay *et al.*, 1997). The bacteria may act as plant growth promoters by fixing atmospheric nitrogen, sequestering iron from the soil and synthesizing phytohormones and enzymes (Gamalero & Glick, 2011; Glick *et al.*, 2007; Hardoim *et al.*, 2008; Reinhold-Hurek & Hurek, 2011; Ryan *et al.*, 2008; Stoltzfus *et al.*, 1997). Nitrogen cycle is very important to bacteria and also to plants. Nitrogen fixation genes are present in different bacteria and it is relevant to know its diversity and how they promote plant growth might be relevant (Bragina *et al.*, 2013). On the other hand, bacterial denitrification reactions (*nirK* and *nirS* genes) produce nitric oxide (NO) which is involved in plant signalling and root elongation (Zeidler *et al.*, 2004). The objectives of this study were to assess the endophytic microbial (bacterial and archaeal) community of *P. pinaster*, with and without PWD, by molecular and cultivation methods, and to define the diversity of bacterial endophytic functional genes involved in nitrogen cycling, mainly in nitrogen fixation (*nifH* and *nif]* genes) and nitric oxide production (*nirK* and *nirS* genes). Cultivation methods were used in order to acquire a collection of organisms for future nematotoxicity assays and to evaluate the microbes' roles in plant metabolism.

2.3. Material and Methods

2.3.1. Sampling areas

Two different areas affected by PWD in Portugal were sample in Coimbra District, Central Portugal: Malhada Velha, Arganil (M) and Avô, Oliveira do Hospital (A) (Chapter 4; Proença *et al.*, 2010). The areas M and A are affected with PWD since 2008 and include mainly *P. pinaster,* with a sparse number of *P. radiata, Quercus faginea* and *Eucalyptus globulus* trees. The sampling area M included 109 trees: 11 *Quercus faginea*, 7 *Eucalyptus globlus*, 85 *P. pinaster* and 6 *P. radiata*. In this area, 12 *P. pinaste*r were symptomatic/dead and 2 *P. radiata* also showed wilting symptoms. The area A included a total of 116 *P. pinaster*, of which 18 were symptomatic and 15 *Quercus faginea* trees.

2.3.2. Plant material

Sampling was performed in Spring-Summer of 2009. Each sample consisted of pinewood cross-sections from cutted trees or wood obtained by drilling a 5 mm diameter hole to a depth of 10 to 15 cm with a sterilized hand borer (Haglof, Mora, Sweden). Fifty two trees were sampled at the trunk at breast height and at the top of the tree. From area M, a total of 26 trees were sampled – 18 *P. pinaster*, four *P. radiata*, two *Eucalyptus globulus* and two *Querqus faginea*. From area A, a total of 26 trees were sampled – 25 *P. Pinaster* and one *Querqus faginea*. The wood samples were placed in labelled and sealed individual sterilized plastic bags and divided in sub-samples for microbiological analysis and nematode screening. All samples were kept at 4°C and analyzed within 24 h. The diameter of sampled trees was measured at breast height (DBH) and the trees classified into 6 symptom classes based on the symptoms they expressed (Chapter 4; Proença *et al.*, 2010).

The bark and sapwood of each sub-sample were removed under sterile conditions and the wood cut in chips. Then, it was sterilized with 70% ethanol, flamed and cut with a flamed knife in smaller chips that were briefly flamed again. In order to release the bacteria from the wood without bacterial multiplication, 5 g of chips (ca. 1x0.5x0.5cm) were incubated in 250 ml flasks with 100 ml of half concentrated R2A liquid medium (Difco Laboratories), under shaking, in the dark, at 25°C, for 2 h. The resulting suspensions were used to assess the microbial community by cultivation-independent methods - Denaturing Gradient Gel Electrophoresis (DGGE), and cultivation methods

- heterotrophic plate counts, followed by Random Amplified Polymorphic DNA (RAPD) typing of selected colonies.

2.3.3. Nematode screening and identification

The presence of nematodes was screened in all sampled trees. Nematodes were extracted, from triplicates of 20 g of each cross-section and 5 g of drilling cores, using modified Baermann funnels (Abrantes *et al.*, 1976). After 48 h, the suspensions were collected and observed using an inverted stereomicroscope. The identification of PWN and other nematodes was based on diagnostic morphological characters.

2.3.4. Endophytic microbial community by cultivation methods

Dilutions of the suspensions obtained as described above were plated in R2A agar (Difco). Plates were incubated up to five days, in the dark, at 25°C. The number of colonies per plate was counted using a stereomicroscope, and the number of colonies per gram of wood was assessed. All colonies from each tree sample with different morphologies were isolated, and preserved in LB medium with 15% glycerol stocks at -80°C after subculture and purification.

DNA from each bacterial isolate was extracted according to Nielsen *et al.* (Nielsen *et al.*, 1995) and isolates were grouped by RAPD typing. RAPD fragments were amplified by PCR, using primer OPA-03 (Table 2.1) as previously described (Chapter 4; Proença *et al.*, 2010). DNA profiles for isolates were grouped on basis of visual similarities of the fragments analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Reproducibility of the patterns was confirmed.

2.3.5. Endophytic microbial community and their functionality by DGGE-analysis

Microbial community DNA of each tree wood sample was extracted, after ressuspension in R2A liquid medium as described above, following the method of Nielsen *et al.* (Nielsen *et al.*, 1995) with an extra incubation step with 1% CTAB (cetyl trimethyl ammonium bromide)/0.7 M NaCl at 65°C for 20 min. DNA was used as template for DGGE analysis of the 16S rRNA gene sequences from Bacteria and

Archaea as well as for functional genes (Table 2.1). The PCR reaction mix (50 μl) contained: reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3), 100 μM (each) deoxynucleoside triphosphates (Invitrogen), 0.2 μM (each) primer, 1.5 U Taq polymerase (Bioline) and 6 μL of purified DNA solution as template.

The 16S rRNA amplicons, obtained by using primer pairs 27F and 1525R for the first PCR, were subsequently used as DNA template for Nested-PCR by using 341F-GC clamp and 907R primers set (Table 2.1). Archaeal analysis was performed by using 21F and 958R for the first PCR, followed by Nested-PCR with Arch915R-GC clamp and Arch519F primers set (Table 2.1).

Functional genes *nifH*, *nifJ*, *nirK* and *nirS* were amplified according to the primers set described in Table 2.1 and re-amplified with GC-clamp for DGGE analysis.

Table 2.1. PCR conditions and primer sequences.

Primers and genes	Temperature cycling (30x)	Primer sequences	References
OPA-03	94°C for 60 s; 40 °C for 60 s; 72 °C for 90 s	5'-AGTCAGCCAC-3'	Operon Technologies, USA
16S rRNA gene	94°C for 60 s; 53 °C for 60 s; 72 °C for 90 s	27F: 5'-GAGTTTGATCCTGGCTCAG-3' 1525R: 5'-AGAAAGGAGGTGATCCAGCC-3'	Rainey *et al.* (1992)
341F-907R	94°C for 60 s; 57 °C for 60 s; 72 °C for 60 s	341F: 5'-*CCTACGGGAGGCAGCAG-3' 907R:5'-CCGTCAATTCATTTGAGTTT-3'	Muyzer *et al.* (1993)
21F–958R	94°C for 45 s; 55 °C for 60 s; 72 °C for 90 s	21F: 5'-TTCCGGTTGATCCYGCCGGA-3' 958R: 5'-YCCGGCGTTGAMTCCAATT-3'	DeLong (1992)
Parch519–Arch915	94°C for 30 s; 57 °C for 40 s; 72 °C for 40 s	Parch519F: 5'-CAGCCGCCGCGGTAA-3' Arch915R: 5'-*GTGCTCCCCCGCCAATTCCT-3'	Coolen *et al.* (2004)
nifH gene	95°C for 40 s; 53 °C for 40 s; 72 °C for 60 s	PolF: 5'-*TGCGAYCCSAARGCBGACTC-3' PolR: 5'-ATSGCCATCATYTCRCCGGA-3'	Poly *et al.* (2001)
nifJ gene	95°C for 40 s; 53 °C for 40 s; 72 °C for 60 s	nifJ 2459F: 5'-*CIGGITGYGGIGAAACICC-3' nifJ 2933R: 5'-CCIATRTCRTAIGCCCAICCRTC-3'	Campbell & Cary (2004)
nirK gene	95°C for 40 s; 53 °C for 40 s; 72 °C for 60 s	nirK 876F: 5'-*ATYGGCGGVAYGGCGA-3' nirK 1040R: 5'-GCCTCGATCAGRTYRTGGTT-3'	Henry *et al.* (2004)
nirS gene	95°C for 40 s; 53 °C for 40 s; 72 °C for 60 s	nirS cd3aF: 5'-*AACGYSAAGGARACSGG-3' nirS R3cd: 5'-GASTTCGGRTGSGTCTTSAYGAA-3'	Throbäck *et al.* (2004)

*A 40-bp-long GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3') was attached to the 5'-end of primers to prevent complete melting of the PCR products during DGGE (Muyzer *et al.*, 1993).

2.3.6. DGGE analyses and clone library construction

DGGE was performed using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA). PCR samples were loaded into 8% polyacrylamide gels with a denaturing gradient ranging from 30-70% (100% denaturant is defined as 7 M urea and 40% formamide). Gels were run at 70 V, for 17 h, at 60°C and stained with ethidium bromide. The gels were photographed using Gel Doc XR System (Bio-Rad). The similarity between various microbial communities was compared based on the digitized DGGE profiles using the cluster analyses technique in Quantity One 4.6.6 (Bio-Rad). The bands in each lane were detected manually and compared, allowing matching profiles to be generated. Matching profiles for each lane were used to produce a dendrogram by employing the unweighted pair group method using arithmetic averages (UPGAMA). The selected bands present in samples from symptomatic and non-symptomatic trees were excised and kept in TE buffer 0.1 M, at 4°C.

On the following day, excised DGGE bands were incubated at 65 °C for 30 min and re-amplified, using 6 µL of the mixture as DNA template, by PCR, with the same Nested-PCR primers as described above, but without GC-clamp. In an agarose gel, bands corresponding to the expected size were excised, purified using the JET Quick PCR Purification Spin Kit (Genomed GmbH, Löhne, Germany) according to the manufacturer's instructions, cloned into pGEM-T Easy (Promega) and transformed into *E. coli* DH5α. From each transformation culture, three positive clones were selected and grown in 5 ml of Luria broth (LB) medium (Difco Laboratories) with ampicillin (100 µg/ml), overnight, at 37°C, with shaking. Plasmids were extracted with the JET Quick Plasmid Miniprep Spin Kit (Genomed GmbH) and kept in TE buffer, at -20 °C, before sequencing.

2.3.7. Sequencing of bacterial isolates 16S rRNA gene and DGGE band clones

Amplification of a nearly full-length 16S rRNA gene sequence from bacterial isolates was achieved by PCR with primers 27F and 1525R (Table 2.1). PCR products with 1,500 bp obtained from isolates were purified (described above), and kept at -20°C for further sequencing. The 16S rRNA gene of endophytic strains representing all RAPD groups were sequenced with primer 27F (Table 2.1) and for DGGE band

clones of 16S rRNA, *nirK*, *nirS*, *nifH*, *nif]* genes were sequenced with pUC/M13-F primer (5'-GTTTTCCCAGTCACGAC-3´) by using Macrogen sequencing service (Seoul, South Korea).

2.3.8. Phylogenetic analysis

All sequences from endophytic microbial community were compared with sequences available in the EMBL/GenBank database using BLASTN network services (Altschul *et al.*, 1997) and with sequences in the Eztaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim *et al.*, 2012). Sequences were aligned within the SINA alignment service (Pruesse *et al.*, 2012). Sequences were also checked for chimeric artefacts by using Mallard software (Ashelford *et al.*, 2006). Sequences were included in 16S rRNA-based Living Tree Project (LTP) release 115 database (http://www.arb-silva.de/projects/living-tree/) by parsimony in ARB software version 5.5 (Ludwig *et al.*, 2004). Phylogenetic dendrograms, of sequences from this study and closest reference sequences, were constructed by the randomized axelerated maximum likelihood (RAxML) method with GTRGAMMA model (Stamatakis, 2006) included inside ARB software (Ludwig *et al.*, 2004).

Sequences from DGGE band clones resulting from each functional gene were translated by using BLASTX from NCBI, queried against the EMBL/GenBank and KEGG databases. Sequences were aligned with the MEGA 5.1 software (Tamura *et al.*, 2011). Phylogenetic dendrograms were constructed using the neighbor-joining method and Poisson correction as substitution model included in MEGA 5.1 software. In both cases, bootstrap analysis with 1,000 replicates was used to evaluate the robustness of the phylogeny.

2.3.9. Statistical analyses

The degree of diversity of the cultivable microbial community in symptomatic trees and healthy trees was based on the number of strains belonging to each phylotype (family). PAST 2.08 software (Hammer *et al.*, 2001) was used to calculate the Shannon–Weaver index (*H'*). In the case of DGGE profiles, the Shannon–Weaver index (*H'*) was calculated based on the number of DGGE bands.

Redundancy analysis (RDA) was performed in order to reveal relationships between pine trees, bacterial species and the environmental variables (two sampling areas, PWD

symptom class (Chapter 4; Proença *et al.*, 2010) and presence of *B. xylophilus*) by using the software package CANOCO (version 4.5.1). RDA was accompanied by Monte Carlo permutation tests to evaluate the statistical significance of the effects of the explanatory variables on the species composition of the samples (van den Brink & Braak, 1999).

2.3.10. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the isolates and DGGE band clones reported in this study have been deposited in the NCBI Genbank database under the accession numbers XXX (submitted on 17-2-2014).

2.4. Results

2.4.1. Nematode screening and identification

B. xylophilus was detected in three symptomatic *P. pinaster* trees area M (M24, M47, M67) and 14 symptomatic *P. pinaster* trees from area A (A12, A25, A36, A37, A41, A52, A60, A72, A88, A96, A103, AB20, AB23, AB29). *B. xylophilus* was not detected in non-symptomatic *P. pinaster*, *Quercus faginea* and *Eucalyptus globulus* trees.

2.4.2. Endophytic microbial community by cultivation methods

The Colony-Forming Units per gram of wood (CFU/g) varied from $1.5 \times 10^3 \pm 7.0 \times 10^2$ to $1.5 \times 10^6 \pm 6.3 \times 10^5$ in area M and from $7.1 \times 10^1 \pm 3.7 \times 10^1$ to $2.3 \times 10^7 \pm 7.0 \times 10^6$ in area A. In general, the number of heterotrophic bacteria was higher in area A than in area M. Besides, symptomatic trees showed more CFU per gram of wood than non-symptomatic trees or low level symptomatic trees. In trees with higher value of CFU/g, *B. xylophilus* was detected in both sampling areas. In 15 trees of area M and 6 trees of area A, bacterial colonies were not detected, most of them in healthy condition (s0).

In total, 313 endophytic bacterial isolates were grouped in 277 RAPD group types (data not shown). One hundred and forty four isolates from area M were sequenced, representing 162 bacterial isolates, and from area A, 133 isolates were sequenced, representing 151 bacterial isolates.

Eight bacterial classes were detected in the trees of areas M and A: *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria*, *Flavobacteria*, *Gammaproteobacteria* and *Sphingobacteriia*. The most abundant bacterial class in area M trees was *Gammaproteobateria* (34.0 %), comprising bacteria of families *Xanthomonadaceae*, *Enterobacteriaceae*, *Sinobacteraceae*, *Pseudomonadaceae* (17.9 %, 8.0 %, 4.3% and 3.7%, respectively). The other bacterial Classes identified in this area were *Actinobacteria* (6.8 %), *Alphaproteobacteria* (19.1 %), *Bacilli* (1.2 %), *Betaproteobacteria* (27.8 %), and *Sphingobacteriia* (10.5 %). The least abundant bacterial class was *Flavobacteria* (0.6 %) (Figure 2.1).

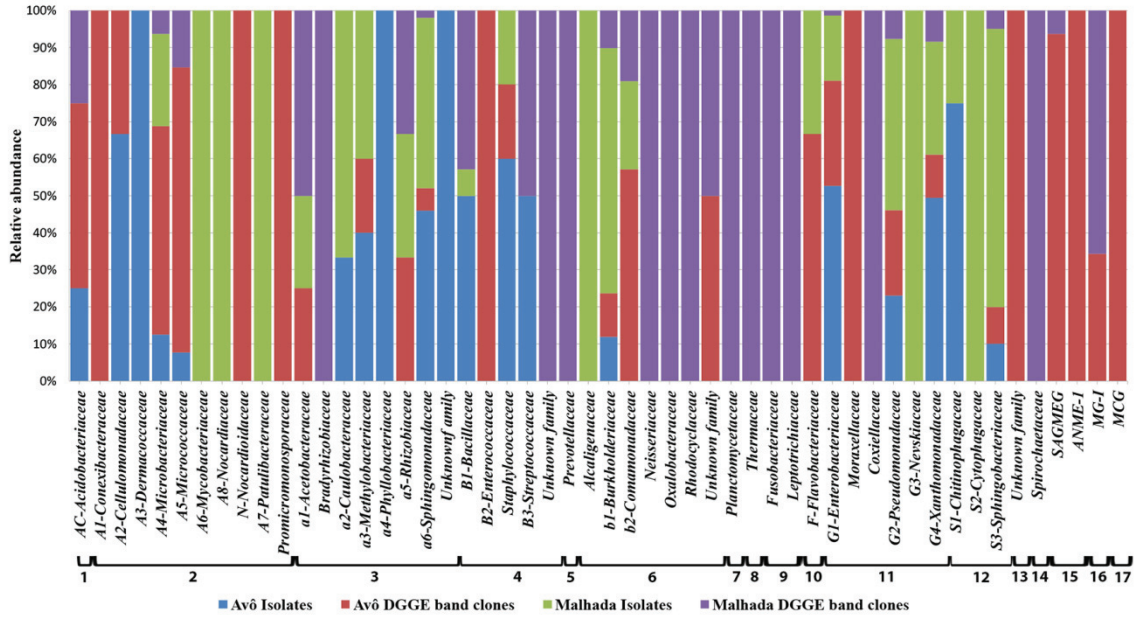

Figure 2.1. Relative abundances and diversities of endophytic microbial communities of pine trees from Malhada (M) and Avô (A) areas based on cultivation and molecular methods. Class- codes: 1- *Acidobacteria*, 2- *Actinobacteria*, 3- *Alphaproteobacteria*, 4- *Bacilli*, 5- *Bacteroidia*, 6- *Betaproteobacteria*, 7- *Planctomycea*, 8- *Deinococci*, 9- *Fusobacteriia*, 10- *Flavobacteriia*, 11-*Gammaproteobacteria*, 12- *Sphingobacteriia*, 13- *Verrumicobiae* and 14- *Spirochaetes*. The phyla of Archaea: 15- *Euryarchaeota* – uncultured archaea of the South African goldmine group (SAGMEG) and uncultured anaerobic methanotrophic archaea (ANME-1), 16- *Thaumarchaeota* – uncultured marine group I (MG-I) and 17- *Crenarchaeota* – miscellaneous *Crenarchaeote* group (MCG). The comprising families of each bacterial class or archaeal groups of each phylum are included in horizontal axis.

In the trees of area A, the most abundant bacterial class was also *Gammaproteobateria* (58.9 %) with members of families *Xanthomonadaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* (31.1 %, 25.8 % and 2.0 %, respectively). The other classes identified in area A were *Actinobacteria* (4.0 %), *Alphaproteobacteria* (20.5 %), *Bacilli* (7.3 %), *Betaproteobacteria* (4.6 %) and *Sphingobacteriia* (3.3 %). The least abundant bacterial class detected was *Acidobacteria* (1.3 %) (Figure 2.1). Strains from the family *Acidobacteria* were only isolated in trees from area A and strains from the family *Flavobacteria* were only isolated from area M trees.

The common species detected in both areas were: *Burkholderia andropogonis*, *B. sordidicola*, *B. xenovorans*, *Herbiconiux solani*, *Luteibacter rhizovicinus*, *Methylobacterium thiocyanatum*, *Novosphingobium resinovorum*, *Pantoea allii*, *Pseudomonas koreensis*, *Pseudoxanthomonas spadix*, *Sphingomonas asaccharolytica*, *Sphingomonas fennica*,

Sphingomonas haloaromaticamans, *Sphingomonas oligophenolica*, *Staphylococcus aureus* subsp. *aureus* (Appendix A). Moreover, by cultivable methods, it was possible to isolate and identify some bacterial species not detected by DGGE. From area M, these species belonged to the families *Mycobacteriaceae* (*Microbacterium profundi*), *Patulibactereaceae* (*Patulibacter minatonensis*), *Nocardiaceae* (*Rhodococcus qingshengii*), *Alcaligenaceae* (*Achromobacter denitrificans/xylosoxidans*), *Nevskiaceae* (*Hydrocarboniphaga daqingensis*), and family *Cytophagaceae* (*Dyadobacter koreensis*); from area A these species belonged to the families *Dermacoccaceae* (*Dermacoccus nishinomiyaensis*), *Phyllobacteriaceae* (*Thermovum composti*); and from both sampling areas the families *Caulobacteraceae* and *Chithinophagaceae* (Figure 2.1 and Appendix A).

2.4.3. Endophytic microbial community analysis by DGGE

The endophytic microbial diversity was also assessed using DGGE profiling (Figures 2.2 and 2.3). Compiled gels (three DGGE gels in each compilation) representing two gels from bacteria and two gels from archaea (one for each area A and M) showed that there were bands repeated in several pine tree samples (Figures 2.2 and 2.3).

From Bacteria DGGE gels of area A, 53 bands were excised. For all areas, three clones of each excised band were sequenced, chimera artifacts identified by Mallard (Ashelford *et al.*, 2006) were removed. The final clones represented the classes *Gammaproteobacteria* (36.4 %), *Actinobacteria* (28.8 %), *Betaproteobacteria* (15.2 %), *Alphaproteobacteria* (6.1 %), *Acidobacteria* (3.0 %), *Flavobacteriia* (1.5 %), *Sphingobacteriia* (1.5 %), *Bacilli* (1.5 %) and *Verrumicrobiae* (0.8 %) (Figure 2.1 and Appendix B). From all the bands that were identified, those that were more often present in the gels were 4, 5, 6, 14, 15, 38, 41, 42, 49, 52 (Figure 2.2a). In the other sampling area, M, 32 bands were excised. These clones represented the Classes *Alphaproteobacteria* (23.8 %), *Betaproteobacteria* (18.8 %), *Gammaproteobacteria* (13.8 %), *Bacilli* (10.0 %), *Actinobacteria* (3.8 %), *Bacteroidia* (3.8 %), *Acidobacteria* (2.5%), *Deinococci* (2.5 %), *Fusobacteriia* (2.5 %), *Planctomycetaceae* (1.3 %), *Sphingobacteriia* (1.3 %), *Spirochaetes* (1.3 %) (Figure 2.1 and Appendix B). From all the bands that were identified, those that were more often present in the gels were 1, 3, 5, 15, 16, 18, 21, 29 (Figure 2.2b). Cloroplast 16S rRNA genes were also found in areas A and M (5.2% and 14.6%, respectively).

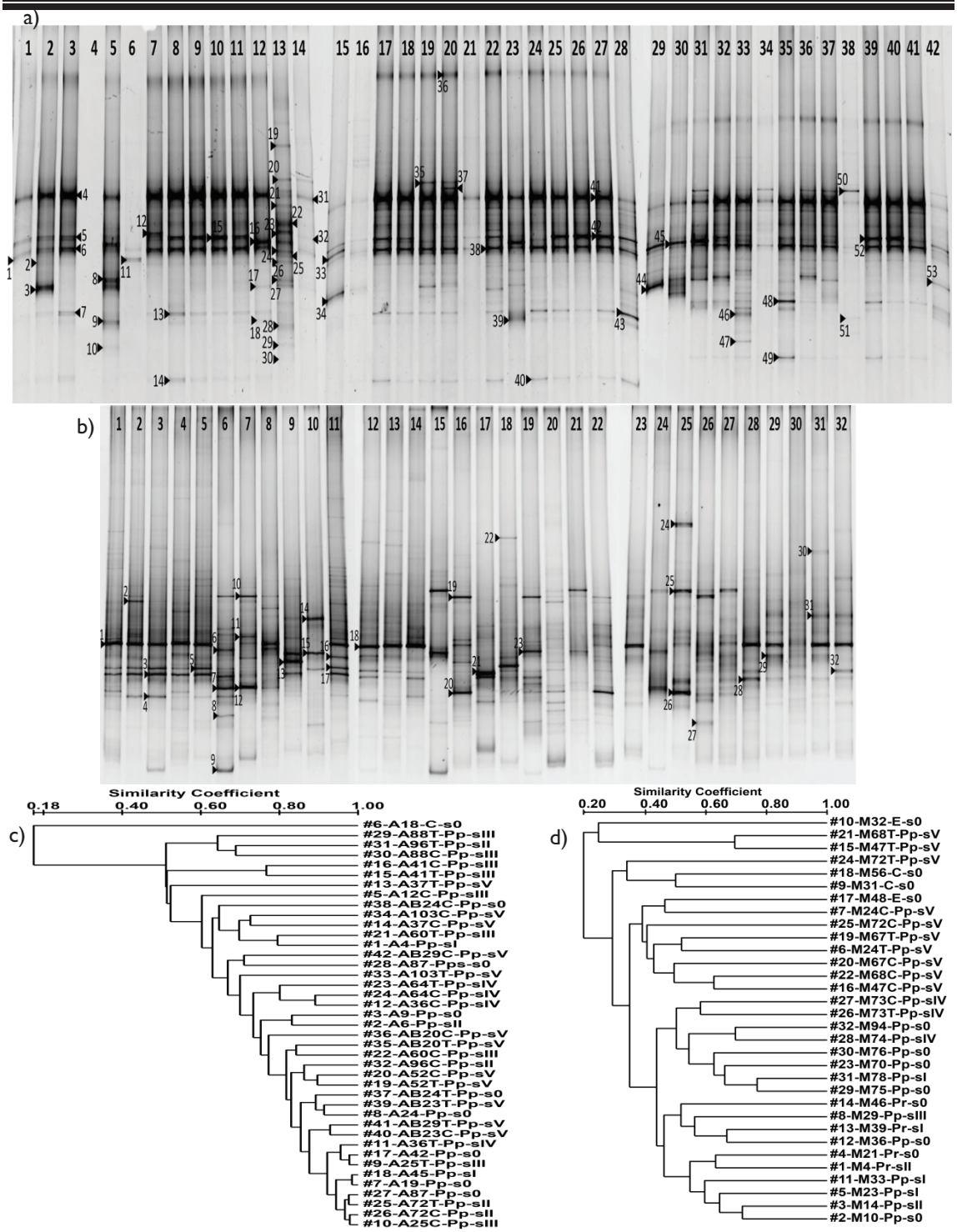

Figure 2.2. DGGE profiles of endophytic bacterial communities from different tree species from Avô (a) and Malhada (b) areas. 16S rRNA gene amplified by 341F-GC clamp and 907R primers. Differences were found between DGGE profiles from trees in different health conditions. Arrows indicate the bands that were excised, cloned and sequenced. Dendrograms based in cluster analysis (UPGAMA) from bacterial DGGE bands showing the similarity coefficient of endophytic bacterial community from different tree species from Avô (c) and Malhada (d) areas. General designation #W#'x-Y-s#" where # - DGGE wells; W – sampling areas of Avô (A/AB) or Malhada (M); #' - cartographic numbers of the trees; x – trunk (T) or crown (C) of trees (without x, means that only trunk was sampled); Y - *Pinus pinaster* (Pp), *Pinus radiate* (Pr), *Quercus faginea* (C), *Eucalyptus globulus* (E); s#" - physiological state of the tree (s0-sV).

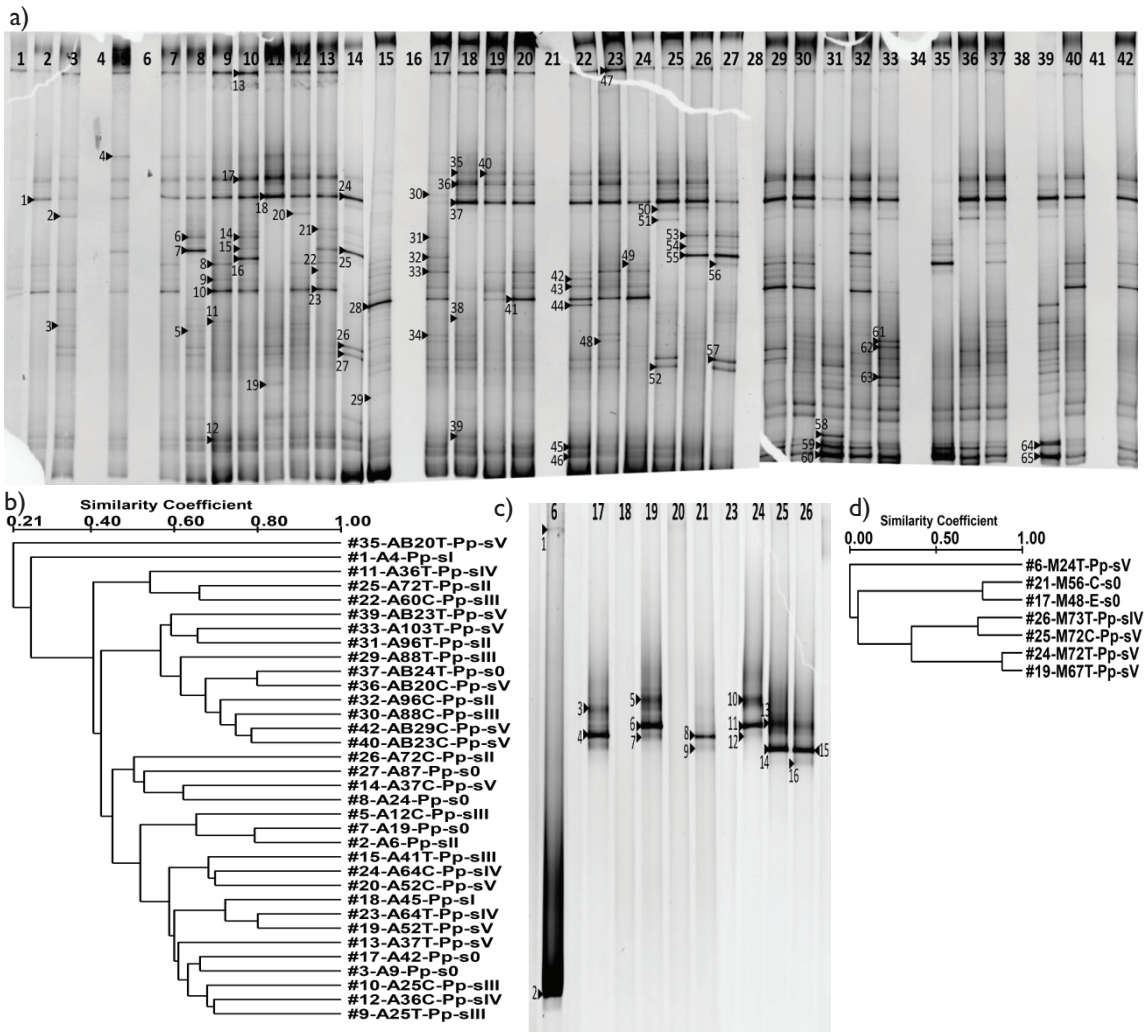

Figure 2.3. DGGE profiles of endophytic archaeal communities from different tree species from Avô (a) and Malhada (c) areas. 16S rRNA gene amplified by 519F-GC clamp and 915R primers. Differences were found between DGGE profiles from trees in different health conditions. Arrows indicate the bands that were excised, cloned and sequenced. Dendrograms based in cluster analysis (UPGAMA) from bacterial DGGE bands showing the similarity coefficient of endophytic bacterial community from different tree species from Avô (b) and Malhada (d) areas. General designation #W#'x-Y-s#" where # - DGGE wells; W – sampling areas of Avô (A/AB) or Malhada (M); #' - cartographic numbers of the trees; x – trunk (T) or crown (C) of trees (without x, means that trunk was sampled); Y - *Pinus pinaster* (Pp), *Pinus radiate* (Pr), *Quercus faginea* (C), *Eucalyptus globulus* (E); s#" - physiological state of the tree (s0-sV).

In total, nine classes were found in area A and 12 classes were found in area M. Comparatively, two classes (*Flavobacteriia* and *Verrumicrobiae*) were only found in A and five classes (*Bacteroidia*, *Deinococci*, *Fusobacteriia*, *Planctomycea* and *Spirochaetes*) were only found in M. The most abundant Classes in A and M areas were

Gammaproteobacteria and *Alphaproteobacteria*, respectively. When comparing bacterial isolates and DGGE band clones, six classes were only found by molecular approaches - *Bacteroidia*, *Deinococci*, *Fusobacteria*, *Planctomycea*, *Spirochaetes* and *Verrumicrobiae*.

From Archaea DGGE gels from area A, 65 bands were excised (Figure 2.3a). These clones represented the phylum *Euryarchaeota* – uncultured archaea of the South African goldmine group (SAGMEG) (55.1 %) and uncultured anaerobic methanotrophic archaea (ANME-1) (10.2 %); the phylum *Thaumarchaeota* – uncultured marine group I (MG-I) (12.3 %) and to the phylum *Crenarchaeota* – miscellaneous *Crenarchaeote* group (MCG) (22.5 %) (Figure 2.1 and Appendix C). From all the bands that were identified, those that were more often present in the gels were 1, 10, 13, 17, 18, 23, 24, 36, 37, 52, 57 (Figure 2.3a). On the other hand, from area M, only 15 bands were excised from 5 *Pinus pinaster* trees in sV symptomatic condition, one *Eucalyptus globulus* tree and one *Quercus faginea* tree (Figure 2.3c). These clones represented the phylum *Euryarchaeota* – uncultured Archaea of the SAGMEG (13.7 %) and the phylum *Thaumarchaeota* – uncultured Archaea of the MG-I (86.3 %). In both sampling areas, all DGGE band clones sequenced showed novel sequences (i.e. < 97 % identity with culturable archaea), suggesting that the endophytic archaeal community of pine trees is novel.

The DGGE profiles from Bacteria or Archaea did not show a specific microbial community profile for pine wilt disease, when using cluster analyses technique in the Quantity One 4.6.6 (Bio-Rad) software: the analysis of the dendrograms of the endophytic microbial community profiles did not group pine trees by symptom classes (Figures 2.2 and 2.3). An endophytic archaeal community was found in *P. pinaster* trees which might play a role in tree metabolism.

2.4.4. Analysis of endophytic genes with proposed metabolic functions

Four functional genes for nitrogen fixation and nitric oxide production were analyzed in this study - *nifH*, *nifJ* and *nirK*, *nirS*, respectively. All these genes were detected from the total DNA of the endophytic microbial community. Sixty-nine clones resulting from 39 DGGE bands were analyzed (data not shown).

The phylogenetic tree of translated sequences of *nifH* gene clones resulted in three clusters: clones from two clusters grouped with the class *Alphaproteobacteria,* mainly

bacteria belonging to *Bradyrhizobium*, *Beijerinckia indica* and *Methylocapsa acidiphila* and the other cluster to *Gammaproteobacteria*, family *Enterobactereaceae* (Figure 2.4).

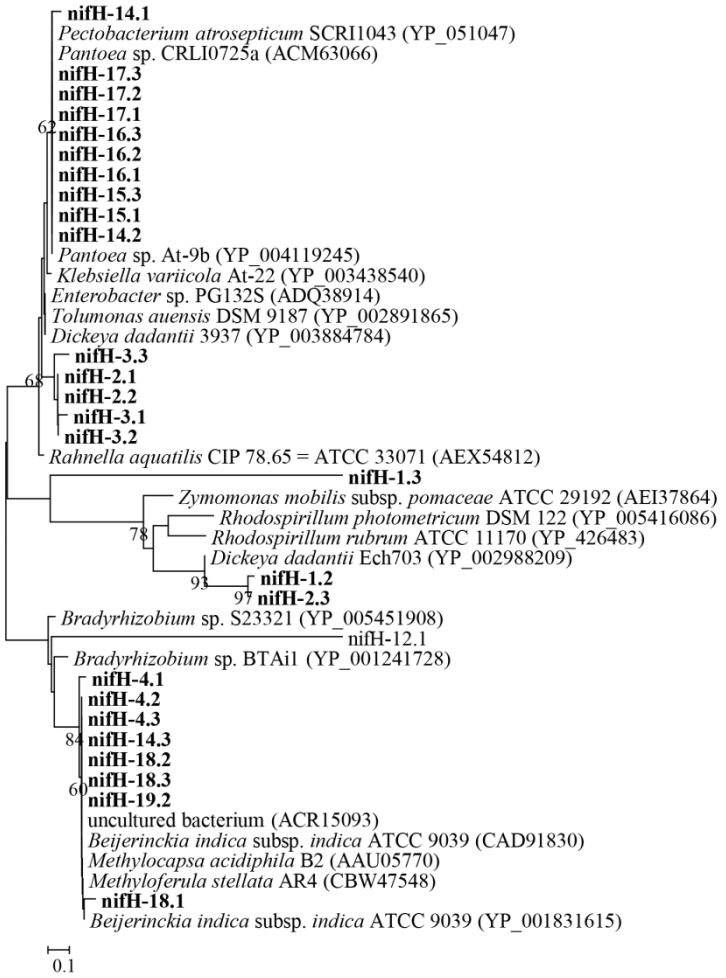

Figure 2.4. Phylogenetic tree of translated sequences of *nifH* gene. The tree was created using the neighbor-joining method and Poisson correction as substitution model included in MEGA 5.1 (Tamura *et al.*, 2011). Sequences generated in this study are indicated in bold and reference sequences were obtained from the NCBI and KEGG databases. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1,000 replications, values below 50% are not shown. Scale bar, 10 inferred nucleotide substitutions per 100 nucleotides.

Phylogenetic analysis of the translated *nifJ* gene showed that all protein sequences were related to the family *Enterobacteriaceae*, mainly belonging to the genera *Rhanella*, *Enterobacter*, *Dickeya* and *Pantoea* (Figure 2.5). Conversion pathways of nitrite to nitric oxide comprise enzymes encoded by *nirK* and *nirS* genes. In this study, the analysis of *nirK* gene showed three main groups, one close to *Pseudomonas entomophila*, a second

close to *Ochrobactrum* and another cluster might constitute a new clade (Figure 2.6). The phylogenetic analysis of *nirS* gene, with the same function as *nirK* gene, clustered with *Alpha-* (*Magnetospirillum*), and *Betaproteobacteria* (*Leptothrix*), as well as *Actinobacteria* (*Arthrobacter*) (Figure 2.7).

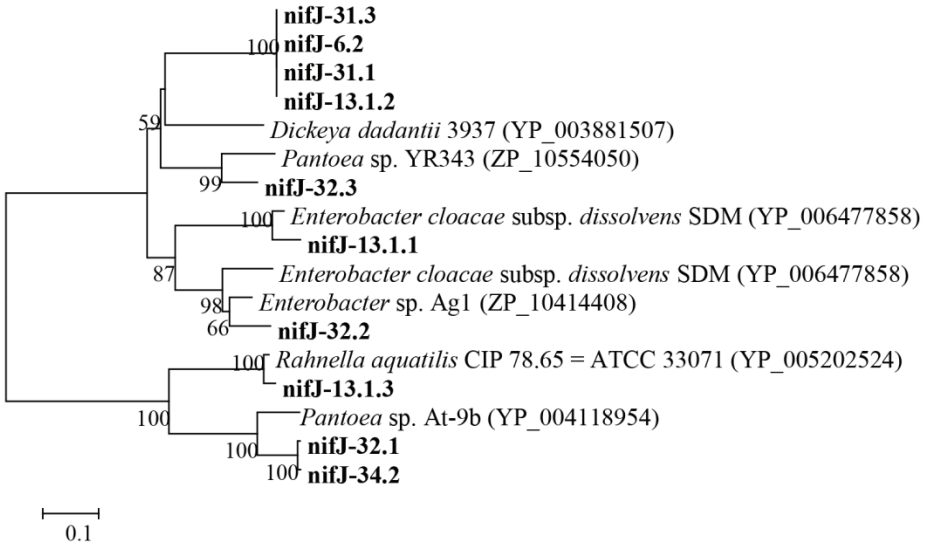

Figure 2.5. Phylogenetic tree of translated sequences of *nifJ* gene. The tree was created using the neighbor-joining method and Poisson correction as substitution model included in MEGA 5.1 (Tamura *et al.*, 2011). Sequences generated in this study are indicated in bold and reference sequences were obtained from the NCBI and KEGG databases. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1,000 replications, values below 50% are not shown. Scale bar, 10 inferred nucleotide substitutions per 100 nucleotides.

The diversity of functional genes seemed to also depend on the sampling area, although the clones included in the *nifJ* clusters were from both sampling areas M and A (Figure 2.5). The *nifH* gene comprised 3 clones from area M (nifH-1.3, nifH-1.2, nifH-2.3) making up a sub-cluster belonging to *Alphaproteobacteria* (Figure 2.4). Regarding the *nirK* gene, the cluster including *Pseudomonas entomophila* and the other cluster including *Ochrobactrum* only had clones from area A (Figure 2.6). As for the *nirS* gene, one of the clusters included exclusively clones from area A (nirS-15.2, nirS-17.3, nirS-19.1, nirS-20.1, nirS-20.2, nirS-20.3) and the other two clusters included clone sequences from area M (Figure 2.7).

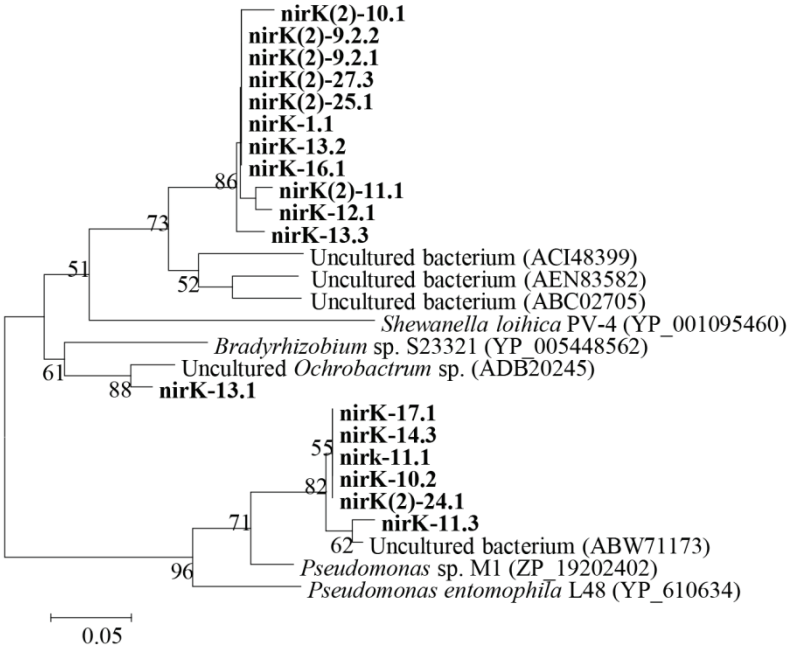

Figure 2.6. Phylogenetic tree of translated sequences of *nirK* gene. The tree was created using the neighbor-joining method and Poisson correction as substitution model included in MEGA 5.1 (Tamura *et al.*, 2011). Sequences generated in this study are indicated in bold and reference sequences were obtained from the NCBI and KEGG databases. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1,000 replications, values below 50% are not shown. Scale bar, 5 inferred nucleotide substitutions per 100 nucleotides.

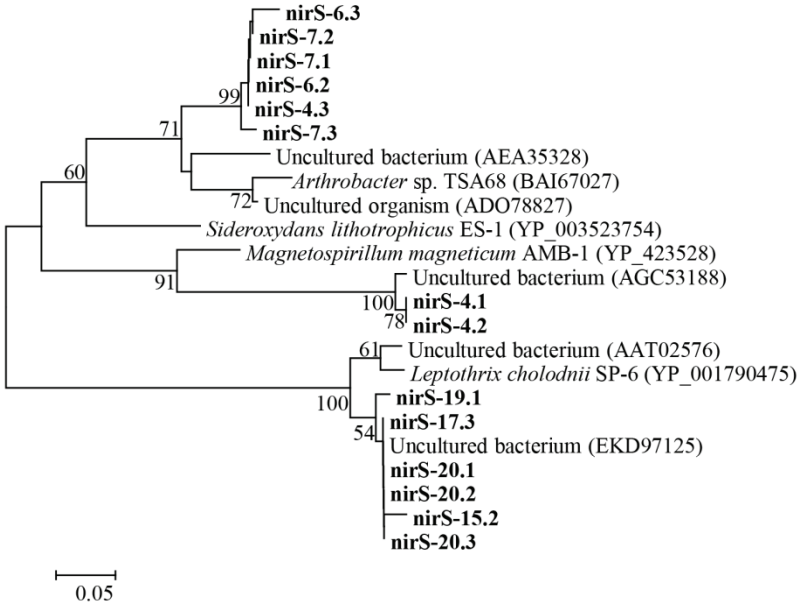

Figure 2.7. Phylogenetic tree of translated sequences of *nirS* gene. The tree was created using the neighbor-joining method and Poisson correction as substitution model included in MEGA 5.1 (Tamura *et al.*, 2011). Sequences generated in this study are indicated in bold and reference sequences were obtained from the NCBI and KEGG databases. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1,000 replications, values below 50% are not shown. Scale bar, 5 inferred nucleotide substitutions per 100 nucleotides.

2.4.5. Statistical analyses

The relationships between pine trees from two different sampling areas, with different health conditions, with or without the presence of *B. xylophilus*, and the bacterial strains isolated were analyzed by RDA (Figure 2.8).

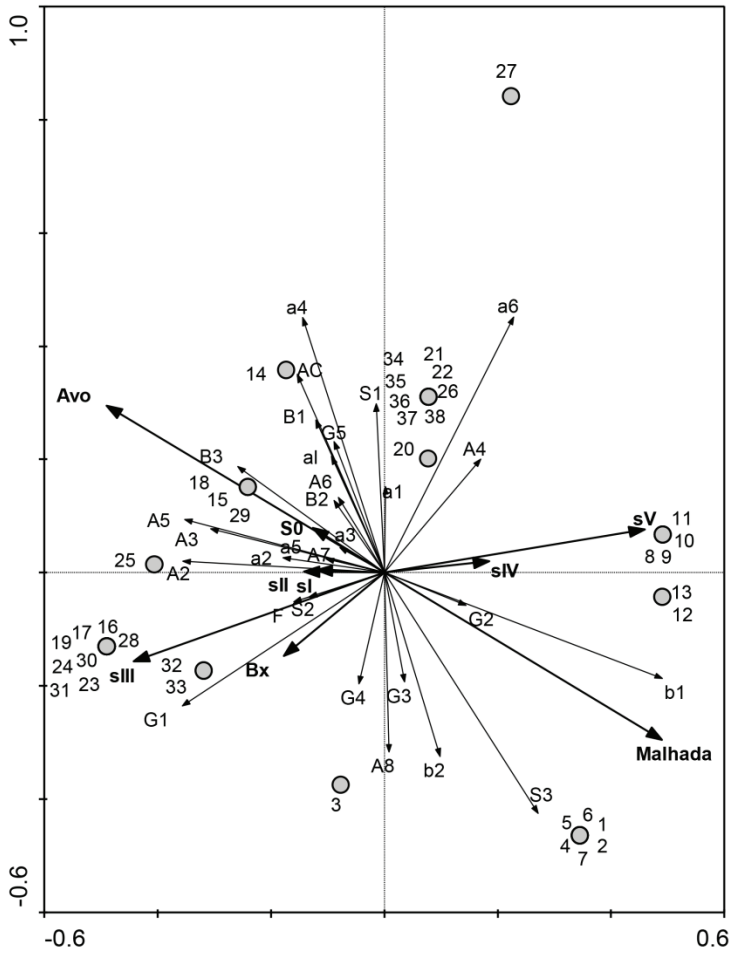

Figure 2.8. Relationships between pine trees, bacterial communities and environmental variables. Redundancy analysis (RDA) performed with endophytic bacterial community from two sampling areas – Avô and Malhada. The cumulative percentage of first and second axis explained 74.0 % of variance. The families included in this analysis are indicated in horizontal axis of figure 1. The environmental variables in bold are: sampling areas (Avô and Malhada), presence of *B. xylophilus* (Bx), PWD symptom classes (s0, sI, sII, sIII, sIV, sV). The numbers are the pine trees sampled 1- M24T-Pp-sV,2- M24C-Pp-sV, 3- M29-Pp-sIII, 4- M47T-Pp-sV, 5- M47C-Pp-sV, 6- M67T-Pp-sV, 7- M67C-Pp-sV, 8- M68T-Pp-sV, 9- M68C-Pp-sV, 10- M72T-Pp-sV, 11- M72C-Pp-sV, 12- M73T-Pp-sIV, 13- M73C-Pp-sIV, 14- A6-Pp-sII, 15- A9-Pp-s0, 16- A12T-Pp-sIII, 17- A12C-Pp-sIII, 18- A24-Pp-s0, 19- A25T-Pp-sIII, 20- A36C-Pp-sIV, 21- A37T-Pp-sV, 22- A37C-Pp-sV, 23- A41T-Pp-sIII, 24- A41C-Pp-sIII, 25- A45-Pp-sI, 26- A52T-Pp-sV, 27- A52C-Pp-sV, 28- A60T-Pp-sIII, 29- A87-Pps-s0, 30- A88T-Pp-sIII, 31- A88C-Pp-sIII, 32- A96T-Pp-sII, 33- A96C-Pp-sII, 34- A103T-Pp-sV, 35- B23T-Pp-sV, 36- B23C-Pp-sV, 37- B29T-Pp-sV, 38- B29C-Pp-sV.

Trees from which no samples could be retrieved were disregarded for this analysis. The first and second axes explained 43.3 % and 74.0 % of cumulative variation, respectively. It was possible to detect, as expected, a strong correlation between the presence of nematodes and the presence of symptoms in trees. The first and second axes seem to separate the pine trees based on the sampling area and on the nematodes presence, respectively. The RDA analysis revealed the pine tree endophytic communities separated according to the sampling areas and the presence of pinewood nematode. *B. xylophilus* apparently showed a close relationship with families *Enterobacteriaceae* (G1) and *Xanthomonadaceae* (G4).

The relative diversity of the endophytic cultivable microbial community of all pine trees was considered high (*H'* = 2.42). The diversity of the endophytic community in M and A areas was *H'* = 2.39 and *H'* = 2.07, respectively. When considering only the symptomatic trees, symptomatic stage equal or higher than sII where the nematode was detected, the diversity of the endophytic community in both areas was higher (*H'* = 2.34 and *H'* = 2.06, respectively) than for healthy trees, with symptomatic stage equal or lower than sII and without nematodes (*H'* = 1.77 and *H'* = 1.07, respectively).

Shannon-Weaver indices for Bacteria and Archaea were higher for symptomatic trees (*H'* = 2.90 and 3.63, respectively) than for healthy trees (*H'* = 2.67 and 3.49, respectively) when calculated from DGGE profiles of area A. On the other hand, DGGE profiles from pine trees from area M showed that the diversity was higher in healthy (*H'* = 3.38) than in symptomatic trees (*H'* = 3.28), although, Archaea were only found in symptomatic trees (*H'* = 1.79).

2.5. Discussion

Endophytic bacteria exist within the living tissues of probably all plants, but compared with herbaceous plants, remarkably little is known about their ecology in trees, such as pine trees. The presence of endophytic bacteria in pine trees, *Pinus* spp., have been reported previously in *P. contorta* (Bal *et al.*, 2012; Shishido *et al.*, 1995), *P. sylvestris* (Izumi *et al.*, 2008; Pirttilä *et al.*, 2000, 2008; Strzelczyk & Li, 2000) and *P. flexilis* (Carrell & Frank, 2012). In this study, we assessed for the first time the diversity of endophytic microbial community from Portuguese *P. pinaster* trees by cultivation and molecular methods, to detect differences between trees infected and non-infected with *B. xylophilus*. The diversity of some of the functional genes involved in nitrogen cycle of the microbial community was also assessed by DGGE analysis, to understand the role of the community in pine trees.

Previous reports showed the genera *Acetobacter*, *Bacillus*, *Brevibacillus, Brevundimonas, Burkholderia, Cellulomonas, Dyadobacter, Gluconacetobacter, Kocuria, Methylobacterium, Paenibacillus* and *Pseudomonas* as part of the endophytic microbial community of different *Pinus* spp. (Bal *et al.*, 2012; Carrell & Frank, 2012; Izumi *et al.*, 2008; Pirttilä *et al.*, 2000, 2008; Shishido *et al.*, 1995; Strzelczyk & Li, 2000). All of these genera, except *Acetobacter*, *Brevibacillus*, *Kocuria* and *Paenibacillus*, were found in this study as part of the endophytic community of *P. pinaster*.

Several studies relating bacteria and PWD focused on bacteria carried by PWN and identified them as belonging to the genera *Bacillus* and *Pseudomonas* in Japan (Kawazu *et al.*, 1996b; Oku *et al.*, 1980); to the genera *Achromobacter*, *Buttiauxella*, *Ewingella*, *Enterobacter*, *Leifsonia*, *Pantoea*, *Peptostreptococcus*, *Rhizobium*, *Serratia*, *Staphylococcus*, *Stenotrophomonas* and, most frequently, *Pseudomonas* in China (Han *et al.*, 2003; Tian *et al.*, 2010; Wu *et al.*, 2013; Zhao & Lin, 2005; Zhao *et al.*, 2003); and to the genera *Burkholderia*, *Brevibacterium*, *Enterobacter*, *Ewingella* and *Serratia* in Republic of Korea (Kwon *et al.*, 2010).

In Portugal bacteria associated with PWN were mainly of the genera *Pseudomonas* and *Burkholderia*, and of the family *Enterobacteriaceae* (genera *Yersinia*, *Serratia*, *Ewingella*, *Pantoea* and *Erwinia*) (Chapter 4; Proença *et al.*, 2010; Vicente *et al.*, 2011b). These bacteria genera were also found in the present work as part of the endophytic microbial community of pine trees. Moreover, both sampling areas of this study suggest *Gammaproteobacteria* as the most abundant class by cultivable methods. This

was also supported by DGGE methods in the Avô area (A), that shares the same most abundant class, in contrast to the Malhada area (M), in which the most abundant class were the *Alphaproteobacteria*. Nevertheless, by using DGGE, it was not possible to detect a specific microbial community profile in pine trees infected with PWD.

Despite limitations (Muyzer & Smalla, 1998), DGGE continues to be an adequate technique for a rapid assessment of microbial community composition. Therefore, in this study, by sequencing DGGE band clones, it was possible to identify six more bacterial classes comparatively to cultivation methods - *Bacteroidia*, *Deinococci*, *Fusobacteria*, *Spirochaetes*, *Planctomycea* and *Verrumicrobiae*.

The relative abundance of bacterial families differed not only among the two sampling areas but also between pine trees in the same sampling area. Differences between sampling sites might be due to differences in environmental conditions related to water availability, pH and soil composition (Eschen *et al.*, 2010) since the two sites were geographically close.

The role of the endophytic community of pine trees has not been studied. A recent study suggested the ten most important plant pathogenic bacteria according to scientific/economic importance (Mansfield *et al.*, 2012). None of these species were isolated or detected by 16S rRNA gene sequencing in this study, therefore a pathogenic role for the endophytic community was disregarded. On the other hand, species detected in both sampling areas were previously described as associated with plants, having a probable role in plant growth promotion. Literature reports the presence of multiple *nif* nitrogen fixation genes in *B. xenovorans* (Chain *et al.*, 2006); *in vitro* production of 3-indol acetic acid (IAA), by *Luteibacter rhizovicinus* and promotion of root development (Guglielmetti *et al.*, 2013); plant-associated strains of the genus *Methylobacterium* able to use methanol as carbon source (van Aken *et al.*, 2004; Trotsenko *et al.*, 2001), produce phytohormones involved in plant growth promotion (auxins and cytokinins) (Ivanova *et al.*, 2001; Koenig *et al.*, 2002), fix atmospheric nitrogen (Sy *et al.*, 2001) and able to produce adenine ribosides which change the morphology of Scots pine buds and increase its viability (Pirttilä *et al.*, 2008). In this study, the diversity indexes of the culturable endophytic community were higher in symptomatic trees than in healthy trees in both sampling areas. This was also confirmed by DGGE profiling in trees from the Avô area (A), for both Bacteria and Archaea. However, for the Malhada area (M), the diversity indexes of Bacteria DGGE

profiles were higher in healthy trees and Archaea were only found in symptomatic trees.

Moreover, Archaea were for the first time reported in *P. pinaster* and might have a role inside the pine trees. The DGGE band clones of this study had close similarity with uncultured archaeal clones that were previous reported as part of the archaeal community from water samples mainly from lakes, ground waters, aquifer and river estuary (Kan *et al.*, 2011; Takai *et al.*, 2001b; Tiago & Veríssimo, 2012) as well as from marine or estuary sediments (Inagaki *et al.*, 2003; Li *et al.*, 2012) and from soil. Thus, the endophytic archaeal community found here constitutes an unexplored area, indicating that pine trees serve as ecological niches for archaea.

RDA demonstrated that the endophytic microbial community is different according to the sampling area, i.e., pine trees were separated by sampling areas. Moreover, the families *Enterobacteriaceae* and *Xanthomonadaceae* seem to have a relationship with the PWN. These findings are in agreement with our previous results, as these families were found associated to PWN (Chapter 4; Proença *et al.*, 2010).

It has long been known that denitrifying bacteria play an important role in the process of soil nitrogen cycling (Hallin *et al.*, 2012). On the other hand, the atmospheric nitrogen fixation constitutes an important function of bacteria, since plants are unable to fix nitrogen and require this essential element for development. The bacterial conversion process of nitrogen into ammonia for plants allows a symbiotic process between plant and microbe, as plants give some nutrients back to bacteria.

Nitrogenase enzyme is involved in nitrogen fixation and multiple subunits are encoded by the genes *nifH*, *nifD*, and *nifK* (reviewed in (Rubio & Ludden, 2002)), with *nifH* most often studied *in situ* as a marker gene. The *nifJ* gene codes for a Mo-dependent dinitrogenase subtype (Dos Santos *et al.*, 2012). The *nirS* and *nirK* genes both catalyze the reduction of nitrite to the metabolic relevant nitric oxide (NO), do not occur simultaneously in the same strain, and do not demonstrate any functional differences (Levy-Booth & Winder, 2010). In the present study, we found some endophytic bacteria possess of *nifH*, *nifJ*, *nirS* and *nirK* genes. The presence of these bacteria supports the possibility of N_2 fixation (and denitrification) occurrence inside trees. The diversity of *nifH* and *nifJ* genes suggested that the population of N_2 fixing bacteria was diverse within the *Gamma* and the *Alphaproteobacteria*, which were also the main groups detected by 16S rRNA gene sequencing, with some of the clones only detected

in one of the sampling areas. Half of the *nifJ* sequences detected were phylogenetically related to *Dickeya dadantii* suggesting the presence of this often pathogenic bacterial species (Costechareyre *et al.*, 2012), although *D. dadantii* was not isolated or phylogenetically detected by 16S rRNA gene sequencing.

In this study, molecular monitoring of the genes of the denitrification pathway, cytochrome cd1-containing nitrite reductase (*nirS*) and Cu-containing nitrite reductase (*nirK*), had the objective of determining to possibility of bacterial production of NO, a relevant physiological regulator of plant stress. In soil, *nirS* seems to be more diverse than *nirK* (Levy-Booth & Winder, 2010) but inside the pine trees the two genes seemed to be diverse, although phylogenetically related with different bacterial classes present in trees. The existence of one of diverse *nir* genes seemed to be more related with the sampling area (only *nirK* in area A, mostly *nirS* in area M) and not with the physiological status of the trees. The *nirK* sequences from one of the subclusters were very divergent from known sequences of uncultured and cultivated organisms, suggesting the presence of this functionality in organisms not yet described. The presence of *nir* genes suggests that endophytic bacterial community can influence the physiological status of the trees, but the mechanism, which leads to the production of NO inside the plant, needs to be explored.

2.6. Acknowledgments

We thank to CAULE association (*Associação Florestal da Beira Serra*) for their help and sampling authorization. We thank Professor Isabel Abrantes for help on *B. xylophilus* identification. This research was partially supported by *Direcção Regional de Florestas*, *Fundo Florestal Permanente* and *Autoridade Florestal Nacional*, through a national project 'O nemátode-da-madeira-do-pinheiro (NMP), *Bursaphelenchus xylophilus*', by FEDER funds through the *Programa Operacional Factores de Competitividade* – COMPETE and by national funds through the *Fundação para a Ciência* e *a Tecnologia* (FCT), Portugal, under the project PTDC/AGR-CFL/115373/2009. D.N.P. was supported by FCT, graduate fellowship SFRH/BD/61311/2009.

3

Chitinophaga costaii sp. nov., an Endophyte of *Pinus pinaster*, and Emended Description of *Chitinophaga niabensis*

Results published in:
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3.1. Abstract

Bacterial strain A37T2^T was isolated from the endophytic microbial community of a *Pinus pinaster* tree trunk and characterized. Strain A37T2^T was Gram-staining-negative, formed rod-shaped cells that grew optimally at 26-30 °C and at pH 5.5-7.5. The G+C content of the DNA was 46.6 mol%. The major respiratory quinone was menaquinone 7 (MK-7) and the major fatty acids were $C_{16:1}$ *ω5c* and iso-$C_{15:0}$, representing 61.7 % of the total fatty acids. The polar lipids consisted of phosphatidylethanolamine, four unidentified aminophospholipids, one unidentified phospholipid, two unidentified aminolipids and another three unidentified lipids. Phylogenetic analysis based on the 16S rRNA gene sequences showed that strain A37T2^T belonged to the family *Chitinophagaceae* forming a distinct branch with *Chitinophaga niabensis* JS13-10^T within the genus *Chitinophaga*. Strain A37T2^T shared between 92.7 and 95.1 % 16S rRNA sequence similarity with the type strains of the genus *Chitinophaga*. The phylogenetic, phenotypic and chemotaxonomic data showed that strain A37T2^T represents a novel species for which we propose the name *Chitinophaga costaii* sp. nov. The type strain is A37T2^T (=CIP 110584^T, =LMG 27458^T). An emended of the description of the *Chitinophaga niabensis* JS13-10^T is also proposed.

3.2. Introduction

The genus *Chitinophaga* (family *Chitinophagaceae*, phylum *Bacteroidetes*) was established by Sangkhobol & Skerman (1981) to include strains of filamentous, gliding and chitinolytic bacteria. *Chitinophaga pinensis* is the type species of the genus. Kämpfer *et al.* (2006) subsequently reclassified four species formally allocated to the genera *Flexibacter* and *Cytophaga* as *Chitinophaga sancti*, *C. filiformis*, *C. japonensis* and *C. arvensicola*. The genus *Chitinophaga* was also emended by Kämpfer *et al.* (2006) by recognizing that gliding motility and ability to hydrolyse chitin and cellobiose are characteristics possessed only by some strains of the genus; these authors also described a new species *Chitinophaga skermanii*. Since then, the following *Chitinophaga* species have been described: *C. ginsengisegetis* and *C. ginsengisoli* (Lee *et al.*, 2007), *C. terrae* (Kim & Jung, 2007), *C. niabensis* and *C. niastensis* (Weon *et al.*, 2009), *C. rupis* (Lee *et al.*, 2009), *C. eiseniae* (Yasir *et al.*, 2011), *C. oryziterrae* (Chung *et al.*, 2012) and *C. cymbidii* (Li *et al.*, 2013). *Chitinophaga* strains were isolated from soil (Kämpfer *et al.*, 2006; Kim & Jung, 2007; Lee *et al.*, 2009, 2007; Weon *et al.*, 2009), rhizosphere soil (Chung *et al.*, 2012), roots (Li *et al.*, 2013) and vermicompost (Yasir *et al.*, 2011). At the time of writing, 15 species belonging to the genus *Chitinophaga* have been validly named.

3.3. Material and Methods

3.3.1. Bacterial strains and culture conditions

Pinus pinaster trees in Avô, Oliveira do Hospital, Portugal, were sampled and the bark and sapwood of each cross-section were removed under sterile conditions. Strain A37T2^T was isolated from dilutions of 10g of wood chips plated on R2A agar (Difco) incubated at 25 °C, for three days. After subculture and purification, strain A37T2^T was preserved at -80 °C in R2A broth supplemented with 15% (v/v) glycerol.

Chitinophaga niabensis DSM 24787^T was obtained from Leibniz-Institut DSMZ, grown under the same conditions as strain A37T2^T and used as a reference in all tests listed below.

3.3.2. Cell morphology and motility

Cell morphology was examined by phase-contrast microscopy under Leica Diaplan light microscope after growth on R2A agar at 30 °C for 48 h incubation. Gliding motility was observed under the microscope in a fresh broth culture as described by Bernardet *et al.* (2002).

3.3.3. Physiological and biochemical characterization

Growth was tested on tryptic soy agar (TSA, Difco), nutrient agar (NA, Difco) and MacConkey agar (Difco) incubated at 26 °C for 5 days. The temperature range (4, 15, 20, 26, 30, 37, 40, 42, 45 °C) and optimum temperature for growth were examined on R2A agar incubated up to 5 days. Salt tolerance was tested on R2A agar supplemented with 0 to 3 % (w/v) NaCl, in 0.5 % increments, at 30 °C up to 5 days. The pH range for growth was examined at 30 °C in R2A broth adjusted by using 50 mM MES (pH 5-7), HEPES (pH 6-8), TAPS (pH 8-9), and CAPSO (pH 10) over a pH range from 5.0 to 10.0, with intervals of 0.5 pH unit. Gram-staining reaction and the presence of cytochrome oxidase and catalase were determined after 24 h of incubation on R2A agar as described by Smibert & Krieg (1994). The ability to hydrolyse agar, esculin, casein, xylan, gelatin, arbutin, elastin, starch, DNA, chitin, carboxymethylcellulose, xanthine and Tweens 20, 40, 60 and 80 at concentration of 1.0 % (w/v or v/v) on R2A agar, after incubation at 30 °C up to 5 days, was determined as described by Tindall *et al.* (2007). Other physiological properties and enzyme activities were determined using

the API ZYM and API 20NE test strips (bioMérieux), at 37 °C and 30 °C, respectively, according to the manufacturer's instructions. Acid production and single-carbon source assimilation were determined using API 50 CH test strips (bioMérieux) as described by Morais *et al.* (2004), according to the manufactures' instruction. Strain ability to oxidize different carbon sources was assessed using Biolog GN2 MicroPlates, incubated at 30°C. The results were recorded daily for up to 7 days using a MicroPlate reader (Sunrise Xread Plus version: V 4.30). Growth under anaerobic conditions was assessed on R2A agar, incubated in anaerobic chambers (GENbox anaer, bioMérieux). The test for flexirubin type pigments was performed by soaking the cells grown on R2A agar at 26 °C during 48h with 20 % (w/v) KOH (Fautz & Reichenbach, 1980). Congo red adsorption was tested by using R2A-Congo red agar (Congo red 25 mg l^{-1}) incubated at 26 °C during 2 days (Freeman *et al.*, 1989).

3.3.4. Antibiotics resistance characterization

Antibiotic-sensitivity tests were performed by using discs (Oxoid) containing 10 µg penicillin, 30 µg vancomycin, 10 µg streptomycin, 30 µg rifampicin, 30 µg tetracyclin and 30 µg nalidixic acid.

3.3.5. Polar lipids and lipoquinones analyses

Cells for polar lipid and lipoquinone analyses were grown on R2A agar at 30 °C for 48h, harvested and lyophilized. Polar lipids were extracted and two dimensional thin-layer chromatography was performed on silica gel G plates (Merck, 10 x 10 cm, 0.25 mm thickness) using chloroform/methanol/water (65:25:4, by vol.) in the first direction and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) in the second direction (da Costa *et al.*, 2006). Lipoquinones were extracted from freeze-dried cells, purified by thin-layer chromatography and separated by high performance liquid chromatography (da Costa *et al.*, 2011a).

3.3.6. Fatty acid analysis

Cells for fatty acid analysis were grown on R2A agar at 30 °C, in sealed plastic plates, for 48 h (Morais *et al.*, 2004). Fatty acid methyl esters (FAMEs) were obtained from the fresh wet biomass and were separated, identified and quantified using the standard MIS

Library Generation Software (Sherlock Microbial ID System, TSBA 6 database, version 6.0) as described previously (da Costa *et al.*, 2011b).

3.3.7. Measurement of the G+C genomic content and phylogenetic analysis of the 16S rRNA gene sequences

The G+C content of the genome was determined by high-performance liquid chromatography as described by Mesbah *et al.* (1989). The 16S rRNA gene was amplified by PCR and sequenced as described by Morais *et al.* (2004) and aligned against representative reference sequences of the most closely related members, obtained from the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.* (2012) and then aligned by SINA (v1.2.9) using the SILVA SEED as reference alignment (http://www.arb-silva.de/aligner/; Pruesse *et al.* (2012). Evolutionary distances were calculated (Jukes & Cantor, 1969) and phylogenetic dendrograms were constructed using the neighbor-joining (Saitou & Nei, 1987) and maximum-likelihood (Olsen *et al.*, 1994) algorithms included in the ARB software package (Ludwig *et al.*, 2004). Trees topologies were evaluated by performing bootstrap analysis (Felsenstein, 1985) of 1,000 data sets by using ARB software package.

3.4. Results and discussion

The major polar lipids were phosphatidylethanolamine (PE), two unidentified aminophospholipids (APL1, APL2) and one unidentified lipid (UL1). Minor amounts of two other unidentified aminophospholipids (APL3, APL4), one unidentified phospholipid (PL), two unidentified aminolipids (AL1, AL2) and two other unidentified lipids (UL2, UL3) were also present. No glycolipids were detected (Figure 1). Although, strain A37T2^T shared the major polar lipid phosphatidylethanolamine with *C. oryziterrae* YC7001^T, *C. sancti* KACC 13046^T, *C. pinensis* KACC 12763^T (Chung *et al.*, 2012), *C. eiseniae* YC6729^T (Yasir *et al.*, 2011), and *C. rupis* CS5-B1^T (Lee *et al.*, 2009), the other polar lipids exhibited a different profile consisting of several aminophospholipids and unknown lipids.

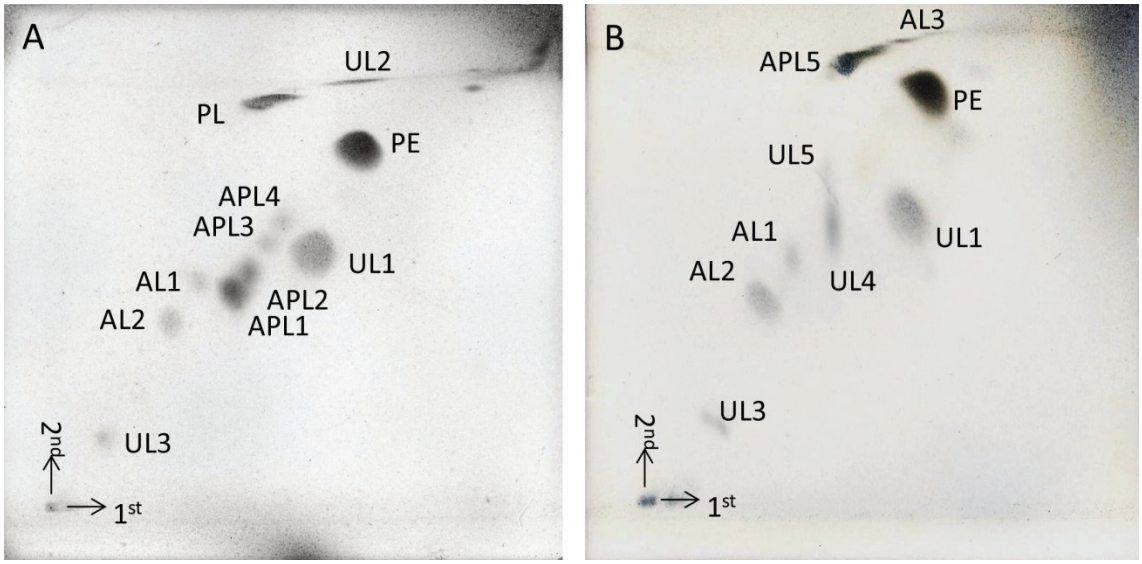

Figure 3.1. Two-dimensional TLC of the total polar lipids of strain A37T2^T (A) and *C. niabensis* DSM 24787^T (B) stained with 5% ethanolic molybdophosphoric acid. PE, phosphatidylethanolamine; APL1-5, unidentified aminophospholipids; AL1-3, unidentified aminolipids; PL, unidentified phospholipid; UL1-5, unidentified unknown lipids.

Menaquinone 7 (MK-7) was the major respiratory quinone of strain A37T2^T, in line with all members of the family *Chitinophagaceae*; minor amounts of other menaquinones were also present.

The major fatty acids of strain A37T2^T were saturated iso-$C_{15:0}$ and unsaturated $C_{16:1}$ *ω*5*c*, which accounted for 61.7% of the total fatty acids (Table 3.1). Significant amounts (≥ 5%) of hydroxylated fatty acids iso-$C_{17:0}$ 3-OH, the summed feature 3

(consisting of $C_{16:1}$ *ω7c* and/or iso-$C_{15:0}$ 2-OH) and iso-$C_{15:0}$ 3-OH were also present in species of the genus *Chitinophaga* (Table 3.1).

Table 3.1. Fatty acid composition of strain A37T2^T and the type strains of related *Chitinophaga* species. Strains: 1, A37T2^T (data from this study); 2, *C. niabensis* DSM 24787^T (data from this study); 3, *C. niabensis* JS13-10^T; 4, *C. sancti* DSM 784^T; 5, *C. filiformis* DSM 527^T; 6, *C. ginsengisoli* Gsoil 052^T; 7, *C. pinensis* DSM 2588^T; 8, *C. oryziterrae* YC7001^T; 9, *C. skermanii* CC-SG1B^T. Data for strains 3-7 and 9 are from Weon *et al.* (2009) and data for strain 8 are from Chung *et al.* (2012). The major cellular fatty acids are in bold. Fatty acids amounting to < 1% in all strains are not shown. Tr, trace amount (< 1%); -, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9
$C_{12:1}$ AT11	-	-	-	4.0	-	-	-	-	-
iso-$C_{13:0}$ 3-OH	-	-	-	-	-	-	-	2.4	-
$C_{14:0}$	1.0	1.1	1.2	1.0	-	-	-	-	2.0
$C_{14:0}$ 2-OH	1.1	-	-	-	-	-	-	-	-
$C_{15:0}$	1.6	-	-	-	-	-	-	-	-
iso-$C_{15:0}$	28.4	44.0	38.2	38.8	41.0	39.0	33.2	33.8	45.8
iso-$C_{15:0}$ 3-OH	5.0	5.0	7.7	4.3	3.2	3.3	3.1	5.4	5.7
$C_{16:0}$	2.5	1.3	2.8	4.6	4.0	1.6	4.1	3.9	3.8
$C_{16:0}$ 2-OH	1.1	1.1	-	-	-	-	-	3.0	-
$C_{16:0}$ 3-OH	1.4	1.9	2.0	-	-	1.2	1.0	-	2.3
iso-$C_{16:0}$ 3-OH	-	-	-	-	-	-	-	1.1	-
$C_{16:1}$*ω5c*	33.3	20.2	19.6	23.9	27.9	31.7	34.7	23.3	19.0
$C_{16:1}$ *ω11c*	-	-	-	3.4	2.7	1.1	3.5	3.6	2.6
$C_{17:0}$ 3-OH	tr	tr	1.6	-	-	-	-	-	-
iso-$C_{17:0}$	3.7	-	-	-	-	-	-	-	-
iso-$C_{17:0}$ 3-OH	8.9	12.4	17.2	7.7	7.8	9.6	11.2	10.3	9.2
iso-$C_{17:1}$ *ω9c*	-	-	-	1.1	-	-	-	-	-
$C_{18:1}$ *ω9c*	-	1.7	-	-	-	-	-	-	-
Unknown fatty acids*									
ECL 13.565	tr	-	-	2.4	1.3	1.6	-	1.5	1.7
Summed features†									
2	1.3	-	-	-	-	-	-		-
3	5.3	9.7	4.4	9.9	8.0	9.1	5.5	8.3	5.6
4	1.7	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	2.1	-

* ECL, Equivalent chain-length.

†Summed features are groups of two or more fatty acids that cannot be separated by GLC with MIDI system. Summed feature 2 contains $C_{12:0}$ aldehyde, $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$. Summed feature 3 contains $C_{16:1}$ *ω7c* and/or iso-$C_{15:0}$ 2-OH. Summed feature 4 contains iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B. Summed feature 5 contains anteiso-$C_{18:0}$ and/or $C_{18:2}$ *ω6, 9c*.

Table 3.2. Differential characteristics of strain A37T2^T and the type strains of related *Chitinophaga* species

Strains: 1, A37T2^T (data from this study); 2, *C. niabensis* DSM 24787^T (data from this study); 3, *C. sancti* DSM 784^T; 4, *C. filiformis* DSM 527^T; 5, *C. ginsengisoli* Gsoil 052^T; 6, *C. pinensis* DSM 2588^T; 7, *C. oryziterrae* YC7001^T; 8, *C. skermanii* CC-SG1B^T. All strains are positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase (API ZYM) and gelatin hydrolysis. All strains assimilate (API 20NE and API 50CH) α-D-glucose. All strains except strain 7 are positive for acid production from esculin ferric citrate. All strains are negative (API ZYM) for lipase (C14). All strains are negative for assimilation of (API 20NE and API 50CH) D-mannitol, capric acid, adipic acid, tri-sodium citrate, phenylacetic acid, D-sorbitol and potassium 2-ketogluconate. Data for reference strains are from Weon *et al.* (2009), Kämpfer *et al.* (2006), Lee *et al.* (2007) and Chung *et al.* (2012). +,positive; w, weakly positive; –,negative; ND, not determined.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Colony colour*	Y	Y	GY	GY	Y	Y	Y	Y
Catalase/oxidase	+/+	+/+	–/ND	–/+	+/+	+/+	–/–	+/+
Cell length (μm)	1–8	1–7	2–15	30–80	1.2–1.6	<40	1.0–7.2	1–2
Gliding motility	–	–	+	+	–	+	–	–
Temperature range (°C)	15-45	5-40	ND	ND	15-42	ND	15-40	5-42
Max. NaCl (%, w/v) tolerance	1.0	2.0	1.0	0.3	<1.0	ND	<0.5	ND
pH range	5.5-7.5	6.0-9.0	ND	ND	5.5-8.5	6.0-9.0	4.5-9.0	5.5-10.0
Chitin hydrolysis	–	–	–	+	+	+	+	ND
Nitrate reduction	–	–	+	+	–	+	–	–
Growth at 37 °C	+	+	–	+		+	+	+
Assimilation of (API 50CH) :								
D-Cellobiose	–	+	ND	ND	ND	+	ND	+
L-Arabinose	–	+	+	–	+	+	–	–
Potassium gluconate	w	–	w	–	w	w	–	w
D-Ribose	w	–	–	–	–	–	ND	–
Enzyme activities (API ZYM):								
Urease	+	–	–	–	+	+	–	–
Esterase (C4)	w	w	w	w	w	w	–	w
Esterase lipase C8	–	–	–	–	–	w	w	w
Cystine arylamidase	+	w	+	w	w	w	–	w
Trypsin	–	+	+	+	+	+	+	+
α-Chymotrypsin	w	+	–	–	–	+	–	–
β-glucuronidase	w	w	–	–	–	–	–	–
α-glucosidase	+	+	+	+	+	+	–	+
α-mannosidase	–	–	w	w	w	+	–	–
α-fucosidase	w	w	+	+	+	+	–	+
DNA G+C content (mol%)	46.6	46.0	43.3	45.0	48.4	45.2	41.3	40.7

*Y-yellow; GY-golden yellow.

Although the major fatty acids are similar in all species of the genus, strain A37T2^T was the only strain to possess $C_{14:0}$ 2-OH, $C_{15:0}$, iso-$C_{17:0}$ and the fatty acids of summed features 2 ($C_{12:0}$ aldehyde, $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$) and 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B), comparison with strains in the phylogenetic clade.

The G+C content of the DNA of strain A37T2^T was 46.6 mol%, a value within the range for *Chitinophaga* species (Table 3.2).

The almost complete 16S rRNA gene sequence of strain A37T2^T (1,503 bp) was aligned with those of the type strains of all *Chitinophaga* species and representative members of other genera in the family *Chitinophagaceae* showing this organism to be a new species belonging to the genus *Chitinophaga*. According to the neighbor-joining phylogenetic tree, the closest relatives to strain A37T2^T were *Chitinophaga niabensis* DSM 24787^T (95.1% sequence similarity), *C. sancti* DSM 784^T (94.6%), *C. filiformis* DSM 527^T (94.6%), *C. ginsengisoli* Gsoil 052^T (94.5%) *C. pinensis* DSM 2588^T (94.2%), *C. oryziterrae* YC7001^T (94.0%) and *C. skermanii* CC-SG1B^T (93.8%) (Figure 3.2). The maximum-likelihood tree showed essentially the same topology.

Biochemical and physiological characteristics of strain A37T2^T are summarized in Table 3.2 and in the species description. In contrast to phylogenetically related strains, strain A37T2^T produced urease but was devoid of trypsin activity.

The phenotypic characterization, enzymatic activities, biochemical and physiological characteristics (Table 3.2), the fatty acid profile (Table 3.1) and phylogenetic evidence indicate that strain A37T2^T represents a novel species of the genus *Chitinophaga* for which we propose the name *Chitinophaga costaii* sp. nov. On the basis of new data obtained in this study, an emended description of *Chitinophaga niabensis* is also proposed.

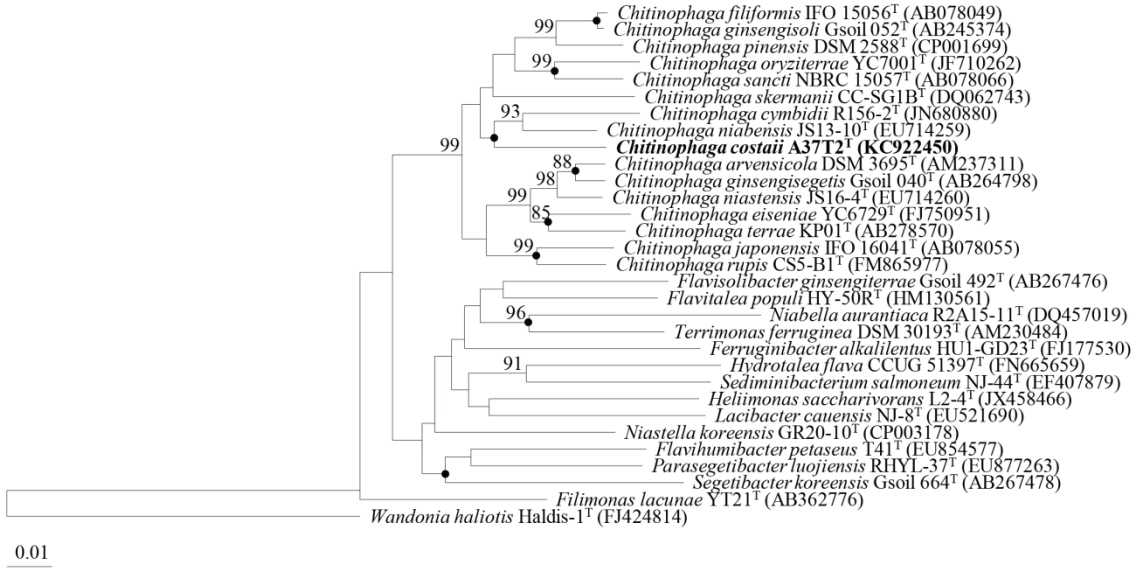

Figure 3.2. Phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequence of strain A37T2^T, other *Chitinophaga* type strains and representatives of the other genera in the family *Chitinophagaceae*. The tree was created using the neighbor-joining method. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1,000 replications; values below 70% are not shown. Symbol (•) indicates node branches conserved when the tree was reconstructed using the maximum parsimony algorithm. The isolate characterized in this study is indicated in bold. Scale bar, 1 inferred nucleotide substitution per 100 nucleotides.

3.4.1. Emended description of *Chitinophaga niabensis* Weon *et al.* 2009

This description is based on that of Weon *et al.* (2009), with the following modifications. In the API 50 CH strip, L-arabinose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, esculin ferric citrate, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose and D-turanose are assimilated; glycerol, D-arabinose, D-xylose, methyl-β-D-xylopyranoside, arbutin, salicin, inulin, D-starch, gentiobiose, L-fucose, potassium 2-ketogluconate and potassium 5-ketogluconate are weakly assimilated; the remaining substrates are not assimilated. In the GN2 MicroPlate dextrin, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, D-maltose, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, succinic acid mono-methyl-ester, D-glucuronic acid, D,L-lactic acid, succinic acid, bromosuccinic acid, L-alanyl-glycine, L-asparagine, L-aspartic acid, glycyl-L-aspartic acid, L-serine, glycerol, D,L,α-glycerol phosphate and α-D-glucose-1-phosphate are oxidized; Tween 40, acetic acid, α-keto valeric acid, L-alanine, L-glutamic acid, glycyl-L-glutamic acid, L-hydroxyproline and D-glucose-6-phosphate are weakly oxidized; the remaining substrates are not oxidized (Appendix D). The polar lipids consist of phosphatidylethanolamine, one unidentified aminophospholipid, three unidentified aminolipids and four unidentified lipids. No glycolipids are detected.

3.4.2. Description of *Chitinophaga costaii* sp. nov.

Chitinophaga costaii (cos.ta'i.i. N.L. gen. masc. n. *costaii*, of Costa, named after Milton S. da Costa, Portuguese microbiologist, for his contributions to the taxonomy of Bacteria).

Cells are Gram-staining-negative, facultatively anaerobic, non-motile, rod-shaped cells 0.5-1 μm in diameter and 1-8 μm in length, after 48h incubation on R2A agar, and non-spore-forming. Colonies on R2A agar are small, smooth, glistening, convex, circular with regular edges and yellow coloured. Cells are able to grow on R2A and nutrient agars, but not on tryptic soy and MacConkey agars. Growth occurs at 15-45 °C (optimum, 26-30 °C), at pH 5.5-8.0 (optimum, pH 7) and on R2A agar supplemented with 0-1 % (w/v) NaCl (optimum, 0 %). Catalase- and oxidase-positive. Colonies on R2A agar with Congo red are coloured red by adsorption of the dye. Flexirubin type pigments are not formed. Resistant to penicillin (10 μg), vancomycin (30 μg), streptomycin (10 μg), nalidixic acid (30 μg), and susceptible to rifampicin (30 μg). Positive for hydrolysis of aesculin, gelatin, Tween 20, Tween 40 (weak positive) and Tween 60. Negative for hydrolysis of xylan, casein, elastin, arbutin, starch, Tween 80, DNA, chitin, carboxymethyl cellulose or xanthine. Negative for nitrate reduction, indole production and glucose fermentation. Positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase; weakly positive for esterase (C4), β-glucuronidase and α-fucosidase; negative for esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsine and α-mannosidase (API ZYM). Positive for urease and β-galactosidase (API 20 NE). Assimilates the following carbon sources (API 20 NE and API 50 CH): α-D-glucose, D-mannose, *N*-acetyl-D-glucosamine, D-maltose, esculin ferric citrate and potassium 5-keto-gluconate; weakly assimilates potassium gluconate, D-ribose, D-xylose, L-lyxose, D-tagatose and D-arabitol. The other organic substrates included in API 50 CH and API 20 NE are not assimilated. In the API 50 CH strip, acid is produced from D-galactose, D-glucose, D-mannose, *N*-acetylglucosamine, amygdalin, esculin ferric citrate, D-maltose, D-melibiose and gentiobiose; acid is weakly produced from L-arabinose, D-xylose, D-fructose, L-rhamnose, methyl-α-D-glucopyranoside, arbutin, salicin, D-cellobiose, D-lactose, D-saccharose, D-trehalose, D-raffinose, D-starch, D-turanose, D-lyxose, L-fucose and potassium 5-ketogluconate; acid is not

produced from the other substrates. In the GN2 MicroPlate dextrin, D-cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, D-maltose, lactulose, D-mannose, D-melibiose, β-methyl-D-glucoside, sucrose, D-trehalose, turanose, D-gluconic acid, uridine, α-D-glucose-1-phosphate and D-glucose-6-phosphate are oxidized; D-galacturonic acid, α-keto butyric acid, sebacic acid, L-aspartic acid, L-glutamic acid, L-proline, L-threonine, thymidine and phenyethylamine are weakly oxidized; the remaining substrates are not oxidized. The major fatty acids (> 25%) are saturated iso-$C_{15:0}$ and unsaturated $C_{16:1}$ *ω5c*. Significant amounts (≥ 5%) of iso-$C_{17:0}$ 3-OH, the summed feature 3 (consisting of $C_{16:1}$ *ω7c* and/or iso-$C_{15:0}$ 2-OH) and iso-$C_{15:0}$ 3-OH are also present. The major polar lipids are phosphatidylethanolamine, two unidentified aminophospholipids and one unidentified lipid. No glycolipid is detected. The major respiratory lipoquinone is menaquinone 7 (MK-7). The DNA G+C content of the type strain is 46.6 mol%. The type strain A37T2^T (= LMG 27458^T = CIP 110584^T) was isolated as endophytic bacteria from the wood of the trunk of *Pinus pinaster* in Avô, Oliveira do Hospital, Portugal.

3.5. Acknowledgments

We are indebted to Prof. J. P. Euzéby (École Nationale Vétérinaire, Toulouse, France) for the etymology of the new organisms' name. This research was financially supported by Program of National Action for Pine Wood Nematode control: "The disease of Pine Wood Nematode", by *Instituto de Financiamento da Agricultura e Pescas* (IFAP) and *Autoridade Florestal Nacional* (AFN) and by the *Fundação para a Ciência e a Tecnologia* (FCT), Portugal, under the PTDC/AGR-CFL/115373/2009 project. DNP was supported by FCT, graduate fellowship SFRH/BD/61311/2009.

4

Diversity of Bacteria Associated with *Bursaphelenchus xylophilus* and other Nematodes Isolated from *Pinus pinaster* Trees with Pine Wilt Disease

Results published in:

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4.1. Abstract

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, has been thought to be the only causal agent of pine wilt disease (PWD), however, since bacteria have been suggested to play a role in PWD, it is important to know the diversity of the microbial community associated to it. This study aimed to assess the microbial community associated with *B. xylophilus* and with other nematodes isolated from pine trees, *Pinus pinaster*, with PWD from three different affected forest areas in Portugal.

One hundred and twenty three bacteria strains were isolated from PWN and other nematodes collected from 14 *P. pinaster*. The bacteria strains were identified by comparative analysis of the 16S rRNA gene partial sequence. All except one Gram-positive strain (*Actinobacteria*) belonged to the Gram-negative *Beta* and *Gammaproteobacteria*. Most isolates belonged to the genus *Pseudomonas, Burkholderia* and to the family *Enterobacteriaceae*. Species isolated in higher percentage were *Pseudomonas lutea, Yersinia intermedia* and *Burkholderia tuberum*. The major bacterial population associated to the nematodes differed according to the forest area and none of the isolated bacterial species was found in all different forest areas. For each of the sampled areas, 60 to 100% of the isolates produced siderophores and at least 40% produced lipases. The ability to produce siderophores and lipases by most isolates enables these bacteria to have a role in plant physiological response. This research showed a high diversity of the microbial community associated with *B. xylophilus* and other nematodes isolated from *P. pinaster* with PWD.

4.2. Introduction

The importance and potentially devastating impact of tree diseases was recognized early in the 20th century with severe epidemics associated with the introduction of new pathogens to native forest ecosystems. The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Nickle *et al.*, 1981) is the causal agent of the Pine Wilt Disease (PWD). Native to North-America, it was introduced to Japan and has spread into China, Korea and into Europe (Portugal and Spain) (EPPO/OEPP, 2009). The etiology of the disease has not been well understood although PWN was confirmed to be the causative agent (Mamiya & Enda, 1972). In general, browning of the tissues is caused by oxidation of phenols which occurs as a result of cellular disorganization (Pirttilä *et al.*, 2008; Whitaker & Lee, 1995). Since the physiological and histological changes in the diseased trees occur before a rapid increase in the number of nematodes, it is alleged that other participants might be involved in the pathological process (Oku *et al.*, 1980). Bacteria in association with *B. xylophilus* have been proposed to be needed for PWD development in *Pinus thunbergii* (Xie & Zhao, 2008), but although studies were performed on isolates, none of the studies (Han *et al.*, 2003; Xie & Zhao, 2008; Zhao & Lin, 2005; Zhao *et al.*, 2007a, 2000, 2003) included the molecular analysis of the microbial community associated with the nematode. Furthermore, the presence of bacteria in plant tissues (endophytes) is usually recognized as positive for the plant. The term endophyte is defined as 'an organism inhabiting plant organs that at some time in its life can colonize internal plant tissue without causing apparent harm to the host' (Petrini, 1991).

The objective of this study was to evaluate the diversity and stability of the bacterial community associated with *B. xylophilus* and other nematodes isolated from *P. pinaster* with PWD.

4.3. Material and Methods

4.3.1. Sampling areas

Pinus pinaster from three different areas affected by PWD in Portugal were sampled: one located between Alcácer do Sal and Grândola (Z), south Portugal (Setúbal District) and two other areas located in Coimbra District, Central Portugal, Malhada Velha, Arganil (M) and Avô, Oliveira do Hospital (A) (Figure 4.1). The area Z is affected with PWD since 1999 and includes mainly *P. pinaster* and *P. pinea* trees with more than 30-35 years old. In this area, four symptomatic *P. pinaster* trees were sampled together with 4 asymptomatic *P. pinaster* trees. The areas M and A are affected with PWD since 2008 and include mainly *P. pinaster,* with a sparse number of *P. radiata, Quercus* and *Eucalyptus* trees. The area M included 109 trees: 11 *Quercus*, seven *Eucalyptus*, 85 *P. pinaster* and six *P. radiata*. In this area, 12 *P. pinaste*r were symptomatic/dead and two *P. radiata* also showed symptoms. The sampling area A included a total of 116 *P. pinaster* and 15 *Quercus* trees where 18 *P. pinaster* were symptomatic. The presence of nematodes was screened in all symptomatic trees.

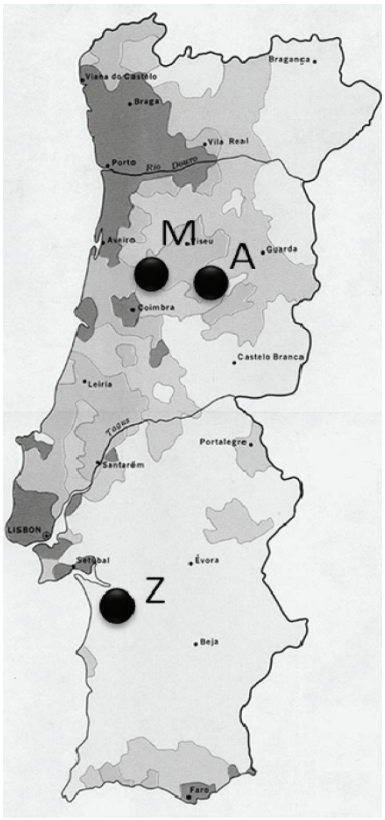

Figure 4.1. Sampling areas. Z - between Alcácer do Sal and Grândola, in the Setúbal District, M - Malhada Velha, Arganil and A - Avô, Oliveira do Hospital.

4.3.2. Plant material

Sampling was performed in Spring-Summer. Each sample consisted of pinewood cross-sections from cut trees or wood obtained by drilling a 5 mm diameter hole to a depth of 10 to 15 cm with a sterilized hand brace drill (Haglof, Mora, Sweden). Fourteen *P. pinaster* trees were sampled at the trunk at breast height and at the top of the tree. The wood samples were placed in labelled and sealed individual plastic bags and divided in sub-samples for microbiological analysis and nematodes screening. All samples were kept at 4°C and analyzed within 24 h. The diameter of sampled trees was measured at breast height (DBH) and the trees classified into 6 symptom classes based on the symptoms they expressed: 0 – tree without symptoms, I - <10% brown leaves, II - 10-50% brown leaves, III – 50-80% brown leaves, IV - >80% brown leaves, V- dead tree without leaves.

4.3.3. Nematode screening and identification

Nematodes were extracted, from triplicates of 20 g of each cross-section, using modified Baermann funnels (Abrantes *et al.*, 1976). After 48 h, the suspensions were collected and observed using an inverted stereomicroscope. The identification of PWN and other nematodes was based on the diagnostic morphological characters. *B. xylophilus* identification was confirmed molecularly by a satellite-DNA species-specific based technique (Castagnone *et al.*, 2005).

4.3.4. Microbial community associated with PWN and with other nematodes

The bark and sapwood of each sub-sample were removed under sterile conditions and the wood cut in ca. 2 cm chips. The wood pieces were placed in Petri dishes with R2A medium and incubated at 25°C, for three days. All bacterial colonies were isolated from the trails made by the nematodes on the medium. In order to isolate bacteria associated only with PWN, wood chips from infected wood samples from area Z were sterilized and processed as previously described and the bacterial colonies were selected only from the trails made by PWN on the medium. Molecular identification of PWN was performed using the satellite-DNA species-specific based technique (Castagnone *et al.*, 2005).

Bacterial isolates were grouped by RAPD typing. RAPD fragments were amplified by PCR, using primer OPA-03 (5' – AGT CAG CCA C – 3') (Operon Technologies, Inc. Alameda, California, USA) together with crude cell lysates. DNA profiles for 123 isolates were grouped on basis of visual similarities of the fragments analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Reproducibility of the patterns was tested.

Nematodes were also extracted from infected wood samples from area Z, identified as *B. xylophilus* on the basis of morphological characters and sterilized with 0.1% sodium hypochlorite and washed with sterilized distilled water. The nematodes were then centrifuged and the resulting nematode pellet was homogenized with sterilized distilled water. DNA was extracted according to Nielsen *et al.* (1995), the 16S rRNA gene from bacteria in the homogenate was amplified by PCR, cloned and sequenced as described below.

4.3.5. 16S rRNA gene sequence of the bacterial isolates from nematode trails and nematode homogenates

Amplification of a nearly full-length 16S rRNA gene sequence from bacterial isolates and from nematodes homogenates was performed by PCR with primers 27F (5' – GAG TTT GAT CCT GGC TCA G – 3') and 1525R (5' – AGA AAG GAG GTG ATC CAG CC – 3') (Rainey *et al.*, 1992). The PCR reaction mix (50 μl) contained: reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3), 100 μM (each) deoxynucleoside triphosphates (Promega, Madison, Wisconsin, USA), 0.2 μM (each) primer and 1.5 U Taq polymerase (Sigma, St. Louis, Missouri, USA). The PCR was performed with 30 cycles: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C.

PCR products with 1500 bp obtained from isolates were purified using the JET Quick PCR Purification Spin Kit (Genomed GmbH, Löhne, Germany) according to the manufacturer's instructions, and sequenced as described below. PCR products from nematode homogenates were cloned into pGEM-T Easy (Promega), transformed into *E. coli* XL1-Blue, extracted, amplified and purified according to standard procedures, and sequenced as described below.

4.3.6. DNA sequence analyses

The 16S rRNA genes from 61 strains associated with nematodes trails and representing all RAPD groups and PCR products from nematode homogenates were subjected to amplification (or re-amplification in the case of the clones) for sequencing. Automated sequencing of the purified PCR products was performed using dRodamina terminator cycle-sequencing kit and ABI 310 DNA Sequencer (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions.

4.3.7. Phylogenetic analyses

All sequences were compared with sequences available in the EMBL/GenBank database using BLAST network services and with sequences in the Ribosomal Database Project II (RDP) (Maidak *et al.*, 2001). Sequences were initially aligned with the CLUSTAL X program (Thompson *et al.*, 1997), visually examined, and relocated to allow maximal alignment. To obtain a more accurate phylogenetic assignment of the OTUs, the aligned sequences were divided into phyla and, in the case of *Proteobacteria*, into classes. Sequences were also checked for chimeric properties by using CHIMERA_CHECK program of RDP (Maidak *et al.*, 2001). The method of Jukes and Cantor (Jukes & Cantor, 1969) was used to calculate evolutionary distances and phylogenetic dendrograms were constructed by the neighbor-joining method using the MEGA 4 package (Tamura *et al.*, 2007).

4.3.8. Siderophore production and proteolytic activity

All isolates were screened for their ability to produce siderophores when cultivated in CAS medium at 25°C during 48h (Schwyn & Neilands, 1987). Strains developing an orange halo were considered as positive. The ability to degrade Tween 20, 40, 60 and 80 at concentration of 1.0% in R2A medium was tested after 3 and 5 days incubation at 25°C. Inoculated medium showing an opaque halo around the zone of growth was considered positive. Skim Milk Agar (R2A:skim milk, 1:1, w/w) was used to detect proteolytic activity. Strains showing a transparent halo around the zone of growth were considered positive.

4.3.9. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the isolates reported in this study have been deposited in EMBL database under the accession numbers from HQ538775 to HQ538819, from FJ784694 to FJ984701, FJ784703 from FJ784705 to FJ784710 and the cloned 16S rRNA genes sequences under the accession numbers FJ784711, and from FJ784713 to FJ784716.

4.4. Results

4.4.1. Nematode screening and identification

Bursaphelenchus xylophilus and other nematodes (Families *Rhabditidae* and *Aphelenchoididae*) were detected in 14 symptomatic trees (class III to V) from the three different affected areas, four from area Z, five from area M and five from area A (Figure 4.1 and Table 4.1). Nematodes of family *Rhabditidae* and *Aphelenchoididae* were found in both symptomatic *P. radiata*.

Table 4.1. Sampled *Pinus pinaster* measured at breast height and classified based on the symptoms they expressed.

Area	*P. pinaster* tree (Code)	DBH (cm)	PWD symptom class [1)]	*Bursaphelenchus xylophilus*	Other nematodes (Rhabditidae and Aphelenchoididae)
A (Oliveira do Hospital)	A12	12	III	+	+
	A25	18.5	III	+	+
	A37	24.5	V	+	+
	A38	23	III	+	+
	AB23	9.5	V	+	+
M (Arganil)	M24	28	V	+	+
	M47	29	V	+	+
	M67	14	V	+	+
	M68	11.5	V	-	+
	M72	8.5	V	-	+
Z (Grândola)	Z1	ND	ND	+	+
	Z2	ND	ND	-	+
	Z3	ND	ND	+	+
	Z5	ND	ND	+	+

ND = Not Determined
DBH = Diameter Breast Height
[1)] PWD symptom classes:
0 - tree without symptoms
I - ‹10% brown leaves
II - 10-50% brown leaves
III - 50 - 80% brown leaves
IV - › 80% brown leaves
V – dead tree without leaves.

4.4.2. Microbial community associated with PWN and with other nematodes

Nematode-associated bacteria were isolated from samples of 11 *P. pinaster* with PWN (Table 4.1). One hundred and twenty three strains were isolated from nematode trails (Figure 4.2).

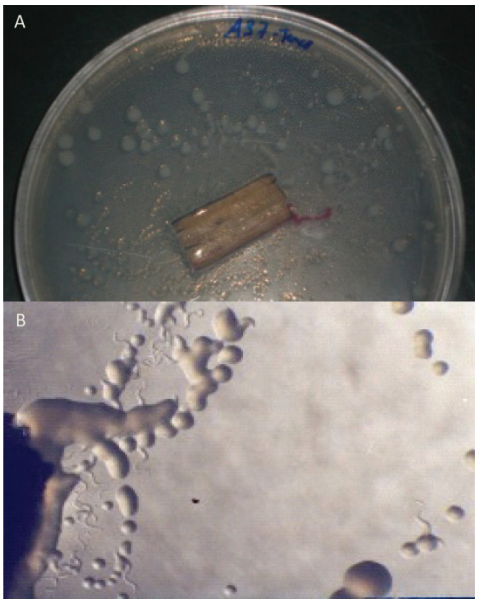

Figure 4.2. Bacterial colonies from nematode trails. Infected wood piece placed on Petri dish with R2A medium and incubated at 25°C, for three days (A). Bacterial colonies from the trails made by the nematodes on the medium were selected for characterization (B).

The number of isolated bacteria and the species isolated were not related with the symptomatology or with the DBH of trees. These strains were grouped into 61 RAPD-types on basis of visual similarities. These strains belonged to two different phylogenetic groups: *Betaproteobacteria* and *Gammaproteobacteria* and one strain belonged to the *Actinobacteria* (family *Microbacteriaceae*) (Figure 4.3). The strains of the *Betaproteobacteria* belonged to the genus *Burkholderia* (19.7%) and one strain was identified as *Janthinobacterium agaricidamnosum*, of the family *Oxalobacteriaceae* (ZR1-2). The isolates belonging to the *Gammaproteobacteria* were identified as strains of the family *Enterobacteriaceae* (41.0%), family *Pseudomonadaceae* (34.4%) and one strain was identified as *Luteibacter rhizovicinus* (*Xanthomonadaceae*) (M24-cE1).

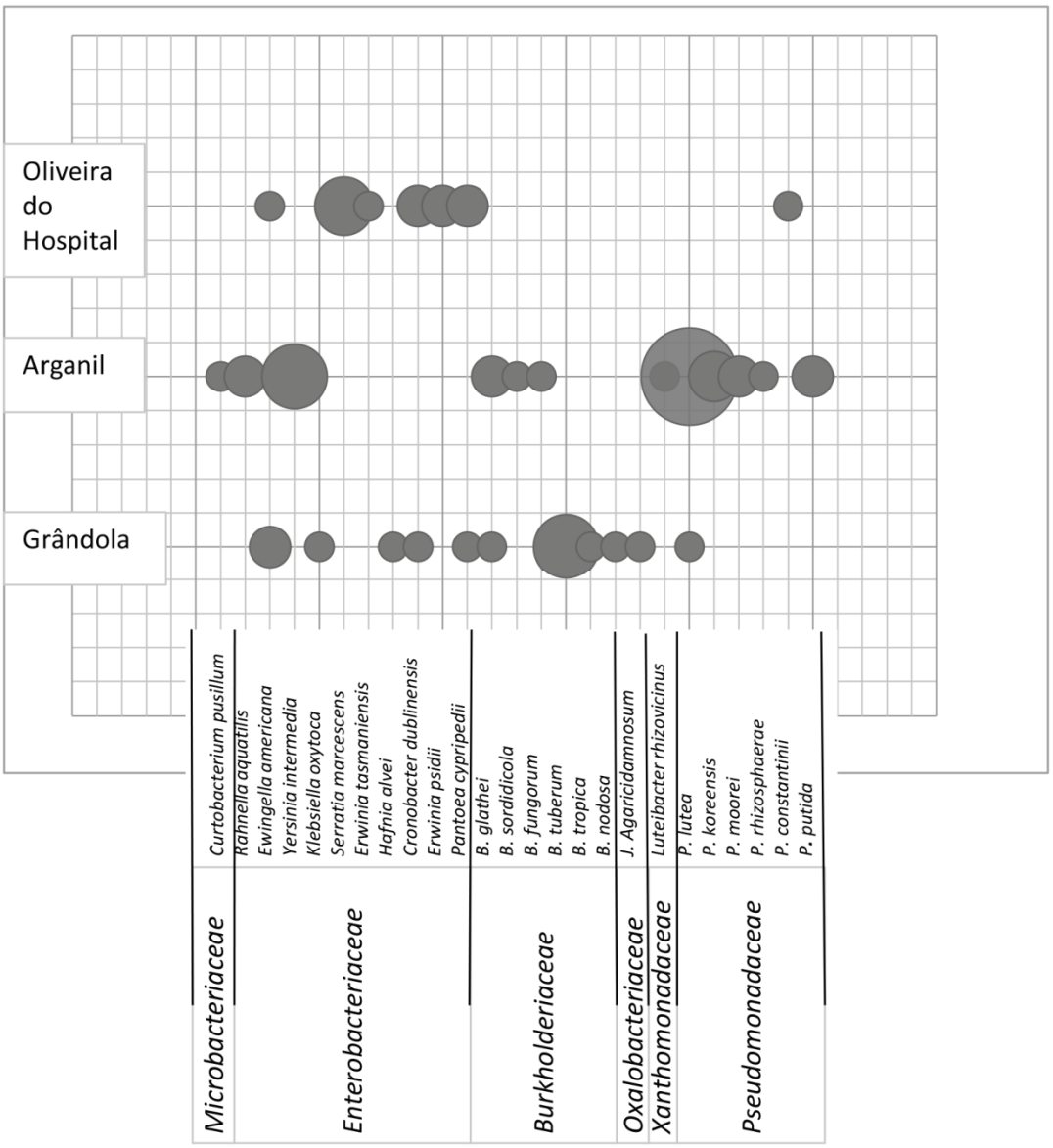

Figure.4.3. Site-dependent microbial diversity associated with PWN. Microbial community associated with the pinewood nematodes, isolated from the three forest areas (A-Oliveira do Hospital, M-Arganil and Z-Grândola) where Pine wilt disease was detected.

The family *Enterobacteriaceae* was represented by ten different species (Figure 4.4A). The most abundant species were *Yersinia intermedia* (20%) and *Serratia marcescens* (16%). The genera *Pantoea*, *Cronobacter*, *Erwinia* and *Ewingella* each included 12% of the isolates. The family *Burkholderiaceae* was represented by six species but the majority of the strains belonged to the species *B. tuberum* (38% of the *Betaproteobacteria*) (Figure 4.4B). The second most abundant species was *B. glathei* (23%). The family *Pseudomonadaceae* was represented by 6 species with the most abundant species being *Pseudomonas lutea* (57%) (Figure 4.4C).

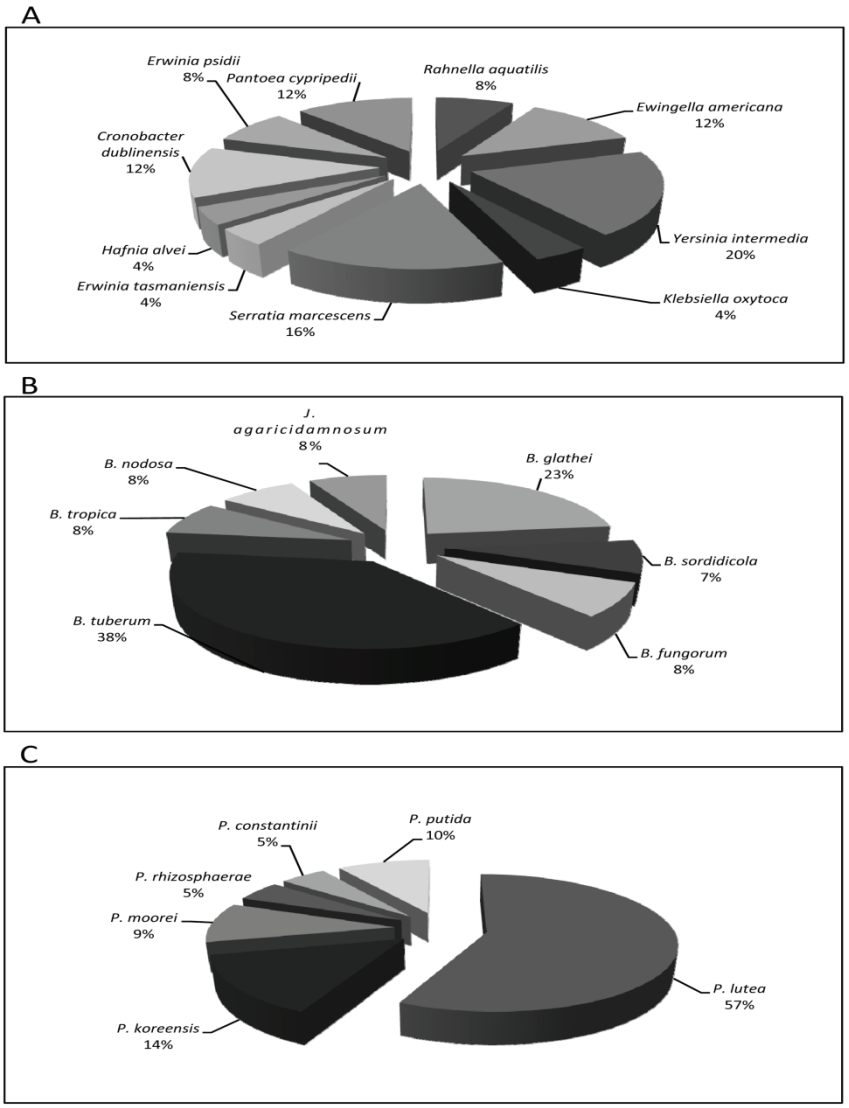

Figure 4.4. Microbial diversity of bacteria carried by *B. xylophilus* and other nematodes isolated in Portugal. Phylogenetic relationship of partial 16S rRNA gene sequences from isolated bacteria with selected reference sequences from identified bacteria in the database were obtained. A – *Gammaproteobacteria* classe - *Enterobacteriaceae* family; B – *Betaproteobacteria* classe *Burkholderiaceae* and *Oxalobacteriaceae* families; C - *Gammaproteobacteria* classe - *Pseudomonadaceae* family.

The bacterial isolates associated only to *Bursaphelenchus xylophilus* isolated from Grândola (area Z) were identified as belonging to the genus *Burkholderia* (50%) and to the *Enterobacteriaceae* (37.5%) and one strain was identified as *P. lutea*. One strain from the genus *Janthinobacterium* sp. (*Oxalobacteriaceae*) was isolated associated with PWN (Figure 4.3).

Nematodes from Arganil trees (area M) carried bacteria from the genus *Pseudomonas* (59.4%), and *Burkholderia* (12.5%) and from the family *Enterobacteriaceae* (21.9%). The only *Luteibacter* and *Actinobacteria* strains were isolated in this area. In Oliveira do

Hospital (area A), 92.3% of the strains associated with the nematodes belonged to the *Enterobacteriaceae* and only 7.7% to the genus *Pseudomonas*.

No single isolated bacterial species associated with *B. xylophilus* and other nematodes was common to all of the different forest areas samples. The *Enterobacteriaceae* associated with nematodes present in two different areas (M and A) were strains belonging to the species *Ewingella americana*, *Cronobacter dublinensis*, and *Pantoea cypripedii*. Strains from *Burkholderia glathei* and *P. lutea* were, respectively, the only *Burkholderiaceae* and *Pseudomonadaceae*, isolated from two areas (Z and M).

The clones from bacteria obtained from homogenates of disinfected PWN were identified as *Betaproteobacteria* belonging to the genus *Burkholderia* (N8) and *Janthinobacterium* (3-1.2), and as *Gammaproteobacteria* from family *Xantomonadaceae* belonging to the genus *Luteibacter* (strain Z4S-80 (2-1.4)) (Table 4.2). Two clones were identified as Gram-positive, an *Actinobacteria* belonging to the family *Corynebacteriaceae* (3-2.2) and a *Bacilli* belonging to the family *Streptococcaceae* (3-2.4).

Table 4.2. Assignment of taxonomic groups to uncultured bacterial clones from molecular nematode-associated bacteria clone libraries and the closest sequence match in database.

Clone	Bacterial family	Closest relative in database (EMBL accession no.)	Identity (%)	EMBL accession no.
N8	*Burkholderiaceae*	*Burkholderia tropica* strain TAt-0750 (EU723241)	99	FJ784711
2-1.4	*Xanthomonadaceae*	*Luteibacter* sp. Z4S-80 (FJ784633)	100	FJ784716
3-1.2	*Oxalobacteraceae*	Clone 14_H06 (FN421682)	100	FJ784713
3-2.2	*Corynebacteriaceae*	Clone nbw1098f01c1 (GQ054100)	100	FJ784714
3-2.4	*Streptococcaceae*	Clone ncd1053f11c1 (HM344016)	99	FJ784715

4.4.3. Biochemical characterization

All isolates were tested for lipases, proteases and siderophore production (Figure 4.5). Most strains produced siderophores, from 60% of the isolates from Grândola (area Z) to 100% of the isolates from Oliveira do Hospital (area A). Proteases were produced only by the isolates from Oliveira do Hospital (30%) and by one isolate from Arganil

(area M), but lipases were produced by 35 strains (63.6%) from the different forest areas. Lipase activity was best when using Tween 40 as substrate.

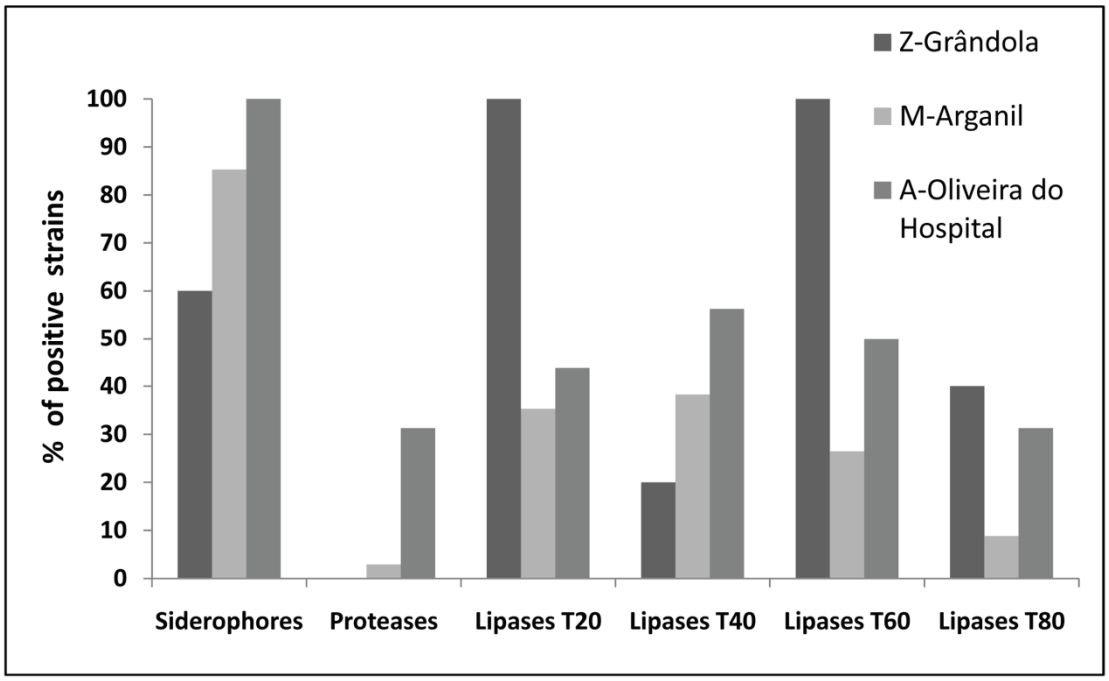

Figure 4.5. Production of siderophores and proteolytic activity of the bacteria isolated. Strains isolated from areas Z-Grândola (■), M-Arganil (■) and A-Oliveira do Hospital (■).

4.5. Discussion

PWD has been detected in Portugal affecting *P. pinaster*. Therefore, it is important to understand first if the PWN isolated from infected trees carries bacteria and, second, what is the diversity of the bacteria associated with PWN and with other nematodes.

Previous works studied bacteria associated with PWN in the perspective that bacteria produce toxins that play an important role in PWD (Zhao & Li, 2008). Bacteria producing toxins to both callus and the seedlings of Japanese black pine (*P. thunbergii*) were described. Zhao and Lin (Zhao & Lin, 2005) described bacteria attached to the surface of PWN and Zhao *et al.* (2000) reported 209 as the average number of bacteria attached to the surface of PWN. Furthermore, authors claimed that healthy *P. thunbergii* did not possess bacteria in the tissues. This was against what was reported by Pirttilä *et al.* (2000) which described the presence of bacteria in *P. sylvestris* tissues.

According to literature the isolates carried by PWN in different countries are mainly species of *Pseudomonas* (in China) and *Bacillus* (in Japan), with both genera in Korea (Zhao & Li, 2008). Furthermore, the number of reported isolated bacterial strains associated with PWN and identified in the literature is low (Wang *et al.*, 2010) and no information exists about differences in diversity of the bacteria carried by PWN or by other nematodes isolated from different forest zones.

In the present study, bacteria strains were isolated from *B. xylophilus* and other nematodes (Families *Rhabditidae* and *Aphelenchoididae*) isolated from *P. pinaster* with different symptom classes and different DBH. The number of isolated bacteria and the species isolated were not related with the symptomatology or with the DBH. The major phylogenetic groups of bacteria isolated associated with PWN and other nematodes present in trees with PWD were *Enterobacteriaceae* and strains from the genera *Burkholderia* and *Pseudomonas*. *Burkholderia* are reported for the first time associated with *B. xylophilus,* although strains from this genus are usually reported as endophytes. Additionally, Gram-positive bacteria associated with PWN were detected by molecular methods and one strain was isolated from nematode trails but none belonged to the genus *Bacillus*. Furthermore, the dominant populations were different according to the different areas. These differences could be explained by differences in the endophytic community of different tree species (from China, Japan, Korea and Portugal) and differences in the soil community. In this work, *Enterobacteriaceae* isolates belonged to different species but the most abundant was the species *Yersinia*

intermedia (with one fifth of the strains), and species *Serratia marcescens*, *Pantoea cypripedii*, *Cronobacter dublinensis*, *Ewingella americana* and species belonging to the genus *Erwinia*. This phylogenetic group was reported to belong to the endophytic community of citrus, cocoa, eucalypti, soybean and sugar cane (Torres *et al.*, 2008). In fact, species *Erwinia tasmaniensis* and *Cronobacter dublinensis* were already reported as beneficial endophytes, while *Ewingella americana, Erwinia psidii* and *Pantoea cypripedii* are reported phytopathogens (Höfte & de Vos, 2006). It is yet unclear if these *Enterobacteriaceae* are connected with the normal flora of *P. pinaster* or with PWD. Within the *Burkholderiaceae*, the species *B. tuberum* was the most common isolate but only carried by nematodes isolated from *P. pinaster* from Grândola (area Z). The presence of this species can most probably be related with its presence in the endophytic community of pine trees since *Burkholderia* species and its diversity were related with plant species and land use management (Salles *et al.*, 2004). The species of the genus *Pseudomonas* have been considered to be in mutualistic symbiosis with PWN and co-responsible for PWD (Guo *et al.*, 2007). Although species of the genus *Pseudomonas* were isolated from nematodes of all sites, the results of this study do not point to the possibility of a *Pseudomonas* species existing associated with PWN in Portugal, as none of the isolated *Pseudomonas* species was found in all 3 sampling areas. In fact, with the exception of *P. lutea*, which was isolated from nematodes of two different sampling areas, all the other isolated *Pseudomonas* species were different from area to area. Moreover, molecular techniques did not allow the detection of *Pseudomonas* in surface-disinfected nematodes. All species identified were described already associated with plants or from soil or even carried by insects. The type strain of *P. lutea* is able to solubilize phosphate (Peix *et al.*, 2004) but *P. constantinii* is described as plant pathogenic bacteria (Höfte & de Vos, 2006). Therefore additional research is needed in order to find out which is the role of these bacteria when associated to PWN and/or inside the plant.

The microbial community associated with Portuguese PWN and with other nematodes, produced siderophores and different lipases and did not produce proteases (casein hydrolysis). Plant lipases have been described as playing a role in plant defense (Kwon *et al.*, 2009). Furthermore, the product of Tween 20 and 80 hydrolysis have been reported to be of essential importance in the regulation of a plant

defense response (Hunzicker, 2009). Therefore, the lipase ability of the bacterial community could have a role, to be explained, in activation of plant defense.

This study contributes to the characterization of the diversity of the microbial community associated with *Bursaphelenchus xylophilus* and other nematodes present in *Pinus pinaster* with PWD. The majority of the strains isolated belonged to phylogenetic groups usually isolated as endophytic bacteria. Further work will be needed to understand the role of these bacteria in PWD.

4.6. Acknowledgments

We thank Manuel Mota as the coordinator of the research line 3 of the national project "O nemátode-da-madeira-do-pinheiro (NMP), *Bursaphelenchus xylophilus*". The authors thank Eng. José Manuel Rodrigues and Eng. Luís Caparica for the *Pinus pinaster* wood samples.

5

Bacteria Carried by Pinewood Nematode in Nebraska, USA: Diversity and Comparison with Isolates from Other Countries

Results submitted in:
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5.1. Abstract

Pine wilt disease (PWD) is native to North America and has spread to Asia and Europe. Lately, mutualistic relationship has been suggested between *B. xylophilus* (PWN), the causal nematode agent of PWD, and bacteria. PWN from infected trees, studied in different countries, were reported to carry bacteria from several genera, however no data exists for the United States. The objective of this study was to evaluate the diversity of the bacterial community carried by *B. xylophilus*, isolated from different *Pinus* spp. with PWD in Nebraska, United States. The bacteria carried by PWN belonged to *Alphaproteobacteria* (1.7 %), *Bacilli* (5.0 %), *Betaproteobacteria* (11.7 %), *Flavobacteriia* (1.7 %) and *Gammaproteobacteria* (79.9 %). Strains from the genera *Chryseobacterium* and *Pigmentiphaga* were found associated with the nematode for the first time. These results were compared to results from similar studies conducted from other countries of three continents in order to assess the diversity of bacteria with associated with PWN. The isolates from the United States, Portugal and China belonged to 25 different genera and only strains from the genus *Pseudomonas* were found in nematodes from all countries. The strains from China were closely related to *P. fluorescens* and the strains isolated from Portugal and USA were phylogenetically related to *P. mohnii* and *P. lutea*. In conclusion, nematodes from the different countries are associated with bacteria of different species suggesting unspecific relationship. Moreover, the diversity of the bacteria carried by the pinewood nematode seems to be related to the geographic area and the *Pinus* species. The roles these bacteria play within the pine trees or when associated with the nematode, might be independent of the presence of the nematode and only related on the bacteria's relationship with the tree.

5.2. Introduction

Pine Wilt Disease (PWD) is caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, isolated in 1931 (Nickle *et al.*, 1981), until now, the only known causal agent of disease. Native to North America (USA and Canada) and only isolated again in a dying tree in 1979, it was introduced to Japan at the beginning of 20th century and it has spread to China, Korea, Taiwan and recently to Europe (Portugal and Spain) (EPPO/OEPP, 2009). PWN is causing one of the most devastating diseases in genus *Pinus* and has caused environmental and economic losses totaling multi-million US dollars around world (Tóth, 2011). The susceptible tree hosts to PWN are mainly conifers of the genus *Pinus*, with Pinus-species differing between geographical locations, such as *P. bunjeana*, *P. densiflora*, *P. luchuensis*, *P. massoniana* and *P. thunbergii* for Far Eastern countries and *P. nigra*, *P. sylvestris* and *P. pinaster* for European species (Evans *et al.*, 1996). In Portugal, *P. pinaster* is the only species known to be susceptible to PWN (Rodrigues, 2008). The transmission of *B. xylophilus* from tree to tree is accomplished by insect vectors of the genus *Monochamus* primarily, during the feeding. Different strategies have been used to address the challenge of PWD, for example, the establishment of a breeding program for resistance and tolerance in pines against damage caused by PWN (Ribeiro *et al.*, 2012; Sniezko, 2006) and the understanding of the role of the endophytic bacterial community (Chapters 4 and 6; Paiva *et al.*, 2013; Proença *et al.*, 2010, 2011; Vicente *et al.*, 2012; Wu *et al.*, 2013). The presence of endophytes ('organism inhabiting plant organs that, at some time in its life, can colonize internal plant tissue without causing apparent harm to the host' (Petrini, 1991)) in plant tissues has been recognized for a long time, but a relevant role for this community has been suggested only lately (Rosenblueth & Martínez-Romero, 2006; Ryan *et al.*, 2008). Some studies have indicated that bacteria may play a role in PWD (Chapter 4; Guo *et al.*, 2007; Han *et al.*, 2003; Higgins *et al.*, 1999; Proença *et al.*, 2010; Zhao *et al.*, 2009). Others suggested that bacteria, carried by the PWN, are phytotoxin producers and interact with the nematode. If true, this may presumably result from a long-term co-evolution between the nematode and the bacteria (Paracer & Ahmadjian, 2000; Zhao & Lin, 2005). In different countries affected by PWD, different bacterial genera have been isolated associated with *B. xylophilus*. In Japan, strains from the genus *Bacillus* (Kawazu *et al.*, 1996a) and from the genus *Pseudomonas* (Oku *et al.*, 1980) were identified to be associated with PWN. Moreover, researchers in China isolated strains

from the genera *Pantoea*, *Peptostreptococcus*, *Enterobacter*, *Serratia*, *Staphylococcus*, *Buttiauxella*, *Stenotrophomonas* but the most frequently isolated genus was *Pseudomonas* (Han *et al.*, 2003; Tian *et al.*, 2010; Zhao & Lin, 2005; Zhao *et al.*, 2003). Strains from the genera *Burkholderia*, *Brevibacterium*, *Enterobacter*, *Serratia* and *Ewingella* were found in Republic of Korea (Kwon *et al.*, 2010). Recently, it was shown that in Portugal bacteria associated with PWN were mainly belonging to the genera *Pseudomonas*, *Burkholderia*, and to the family *Enterobacteriaceae* (genera *Yersinia*, *Serratia*, *Ewingella*, *Pantoea* and *Erwinia*) (Chapter 4; Proença *et al.*, 2010). These results were later confirmed by a second study (Vicente *et al.*, 2011b). The methodologies used to identify the bacterial isolates recovered from the nematodes varied between the different studies. Some studies characterized their isolates by cultivation methods and identification kits (Han *et al.*, 2003; Kawazu *et al.*, 1996a; Kwon *et al.*, 2010; Oku *et al.*, 1980; Roriz *et al.*, 2011; Tian *et al.*, 2010; Zhao & Lin, 2005; Zhao *et al.*, 2003) and other sequenced the 16S rRNA genes and queried them with the international databases (Chapter 4; Proença *et al.*, 2010; Roriz *et al.*, 2011; Vicente *et al.*, 2011b; Wu *et al.*, 2013; Zhu *et al.*, 2012). Therefore, identifications of the isolated strains obtained only by biochemical methods may be incomplete, especially for some of the genera, potentially leading to wrong conclusions regarding bacterial communities.

The objective of this study was to evaluate, the diversity of the bacterial community carried by *B. xylophilus* in the United States from different tree species of the genus *Pinus* with PWD. Furthermore, in order to identify a bacterial species that is commonly associated with all *B. xylophilus*, suggesting a privileged relationship with a role in PWD, EMBL/GenBank database information and previous publications were utilized for analysis.

5.3. Material and methods

5.3.1. Sampling areas

Sampling was performed on June of 2011. *Pinus* trees from five different areas affected by PWD in Nebraska, USA were sampled: large golf course (G), small Golf course (GS), Denis Land (D), Walter Land (W) and University of Nebraska-Lincoln East Campus (UNL) (Table 5.1). The sampling areas included pine trees with and without pine wilt disease, except the golf course where all the pine trees were healthy. G and Gs were golf courses with grass, and scattered pine trees from the species *P. sylvestris*. D and W were woodland areas, with different pine tree species. UNL was grass land with trees from different species, in groups. In area G, six symptomatic *P. sylvestris* trees and one asymptomatic *P. sylvestris* tree were sampled. In area GS, two symptomatic *P. sylvestris* trees were sampled. In area D, four symptomatic pine trees, three *P. sylvestris* and one *P. nigra*, and three asymptomatic pine trees, two *P. sylvestris* and one *P. nigra,* were sampled. In area W, one asymptomatic *P. ponderosa* tree and eight symptomatic pine trees, four *P. nigra* and four *P. sylvestris* were sampled. In the UNL area, four symptomatic pine trees, one *P. nigra* and three *P. sylvestris*, and one asymptomatic *P. ponderosa* tree were sampled (Table 5.1).

5.3.2. Plant material

Each sample consisted of pinewood cross-sections from cut trees or wood obtained by drilling a 5 mm diameter hole to a depth of 10 to 15 cm with a sterilized hand brace drill (Haglof, Mora, Sweden). The wood samples were placed in labelled and sealed individual sterile plastic bags. All samples were kept at 4°C and analyzed within 24 h. The trees were classified into 6 symptom classes previously defined (Chapter 4; Proença *et al.*, 2010), based on the symptoms they expressed.

5.3.3. Microbial community carried by PWN

The bark and sapwood of each sample were removed under aseptic conditions and the wood cut in ca. 2 cm chips. The wood pieces were placed onto R2A agar plates and incubated at 25°C, for three days. All bacterial colonies were isolated from the trails made by the nematodes on the medium (Chapter 4; Proença *et al.*, 2010). The R2A agar plates without nematodes were also used for isolation of bacteria. Bacterial

isolates were grouped by RAPD typing. RAPD fragments were amplified by PCR, using primer OPA-03 (5' – AGT CAG CCA C – 3') (Operon Technologies, California, USA) together with crude cell lysates. DNA profiles for 130 isolates were grouped on basis of visual similarities of the fragments analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Reproducibility of the patterns was confirmed.

5.3.4. Nematode screening and identification

Nematodes were removed from R2A medium plates and collected into 250 µL tubes with dH_2O. The nematodes were observed using an inverted stereomicroscope. The identification of PWN and other nematodes was based on their diagnostic morphological characters. Identification of *B. xylophilus* was verified molecularly by a satellite-DNA species-specific based technique (Castagnone *et al.*, 2005).

5.3.5. Determination of 16S rRNA gene sequences of bacterial isolates from nematode trails

Amplification of a nearly full-length 16S rRNA gene sequence from bacterial isolates was performed by PCR with primers 27F (5' – GAG TTT GAT CCT GGC TCA G – 3') and 1525R (5' – AGA AAG GAG GTG ATC CAG CC – 3') (Rainey *et al.*, 1992). The PCR reaction mix (50 µl) contained: reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3), 100 µM (each) deoxynucleoside triphosphates (NZYTech, Lisbon, Portugal), 0.2 µM (each) primer and 1.5 U Supreme NZYTaq DNA polymerase (NZYTech). The PCR was performed with 30 cycles: 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C.PCR products with 1,500 bp obtained from isolates were purified using the NZYGelpure kit (NZYTech) according to the manufacturer's instructions, and sequenced as described below.

5.3.6. DNA sequence analysis and phylogenetic analysis

The 16S rRNA genes, from RAPD-types representing all different strains, were subjected to amplification for sequencing. Automated sequencing of the purified PCR products was performed by Eurofins MWG Operon (California, USA).

All sequences were compared with sequences available in the EMBL/GenBank database using BLASTN network services (Altschul *et al.*, 1997) and with sequences in the

Eztaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim *et al.*, 2012). Sequences were aligned within the SINA alignment service (Pruesse *et al.*, 2012) and checked for chimeric artefacts by using Mallard software (Ashelford *et al.*, 2006). Phylogenetic dendrograms were constructed by the maximum likelihood (RAxML) method included inside ARB software (Ludwig *et al.*, 2004) and also the neighbor-joining phylogenetic trees were constructed by using Jukes-Cantor method (Jukes & Cantor, 1969) included in MEGA 5 software (Tamura *et al.*, 2011). Bootstrap analysis with 1,000 replicates was used to evaluate the robustness of the phylogeny.

All sequences used above were compared with sequences from strains isolated carried by wild *B. xylophilus*, (isolated from trees), present in the databases. A total of 98 sequences were found: 60 from Portugal in 2010 (Chapter 4; Proença *et al.*, 2010); 21 from Portugal in 2011 (Vicente *et al.*, 2011b); 2 from China in 2012 (Zhu *et al.*, 2012) and 15 from China in 2013 (Wu *et al.*, 2013). The sequences were aligned and the dendrograms were constructed as mentioned above.

5.3.7. Data analysis

Relationships between pine trees, bacterial species and the environmental variables (pine trees species including *P. nigra*, *P. sylvestris* and *P. ponderosa*; five sampling areas and PWD symptom class (Table 5.1) and *B. xylophilus* presence) were analyzed by redundancy analysis (RDA) using the software package CANOCO (version 4.5.1).

RDA was accompanied by Monte Carlo permutation tests to evaluate the statistical significance of the effects of the explanatory variables on the species composition of the samples (van den Brink & Braak, 1999).

5.3.8. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the bacterial isolates carried by PWN reported in this study have been deposited in Genbank/EMBL database under the accession numbers KF214941-KF214978.

5.4. Results

5.4.1. Nematode screening and identification

The pinewood nematode *B. xylophilus* was detected in nine symptomatic pine trees (class III and V of PWD symptom class) from three of the five sampling areas (Table 5.1). *B. xylophilus* and nematodes from other families (Families *Aphelenchoididae* and *Rhabditidae*) were found in two symptomatic pine trees (Arv13 and Arv22) from the species *P. nigra* and *P. sylvestris*, respectively. No nematodes were detected in the six asymptomatic trees.

Table 5.1. Sampled *Pinus* spp. from different geographical areas and classified based on the PWD symptoms they expressed. +, presence of nematode; -, absence of nematode.

Sampling Area	Sample Tree	Pine tree species	PWD symptom class[a]	*B. xylophilus*	Other nematodes[b]
Golf course (G)	Arv1	*P. sylvestris*	V	-	-
	Arv2	*P. sylvestris*	0	-	-
	Arv3	*P. sylvestris*	I	-	-
	Arv4	*P. sylvestris*	V	-	-
	Arv6	*P. sylvestris*	II	-	-
	Arv7	*P. sylvestris*	III	-	-
Golf course small (Gs)	Arv8	*P. sylvestris*	V	-	-
	Arv9	*P. sylvestris*	III	-	-
Denis Land (D)	Arv10	*P. sylvestris*	V	+	-
	Arv11	*P. sylvestris*	III	+	-
	Arv12	*P. sylvestris*	III	-	-
	Arv13	*P. nigra*	V	+	A
	Arv14	*P. nigra*	0	-	-
	Arv15	*P. sylvestris*	0	-	-
	Arv16	*P. sylvestris*	0	-	-
Walter Land (W)	Arv17	*P. nigra*	IV	-	-
	Arv18	*P. sylvestris*	IV	-	-
	Arv19	*P. ponderosa*	0	-	-
	Arv20	*P. nigra*	V	+	-
	Arv26	*P. sylvestris*	V	-	-
	Arv27	*P. sylvestris*	IV	-	-
	Arv28	*P. sylvestris*	V	+	-
	Arv29	*P. nigra*	V	+	-
	Arv30	*P. nigra*	V	-	-
UNL East Campus (UNL)	Arv21	*P. ponderosa*	0	-	-
	Arv22	*P. sylvestris*	V	+	R
	Arv23	*P. sylvestris*	V	+	-
	Arv24	*P. sylvestris*	V	-	-
	Arv25	*P. nigra*	V	+	-

[a]0 – tree without symptoms, I - <10% brown leaves, II - 10-50% brown leaves, III – 50-80% brown leaves, IV - >80% brown leaves, V- dead tree without leaves. [b]A- *Aphelenchoidae* and R- *Rhabditidae*.

5.4.2. Microbial community carried by PWN

The microbial community carried by nematodes was isolated from nine pine trees (Table 5.1). A total of 107 strains were isolated of which forty-seven isolates were identified by microscopy as yeasts. Bacterial strains carried by PWNs were found in four *P. sylvestris* trees (Arv-11, -22, -23, and -28) and in four *P. nigra* (Arv-13, -20, -25, and -29) and all the strains were isolated. No bacteria were isolated from other nematodes. The bacterial strains were grouped into 38 RAPD-types on basis of visual similarities, representing a total of 60 bacterial strains. Based on identification using international databases, these bacterial strains belonged to six phylogenetic classes: *Alphaproteobacteria* (1.7 %), *Betaproteobacteria* (11.7 %), *Gammaproteobacteria* (79.9 %), *Flavobacteriia* (1.7 %) and *Bacilli* (5.0 %) (Figure 5.1).

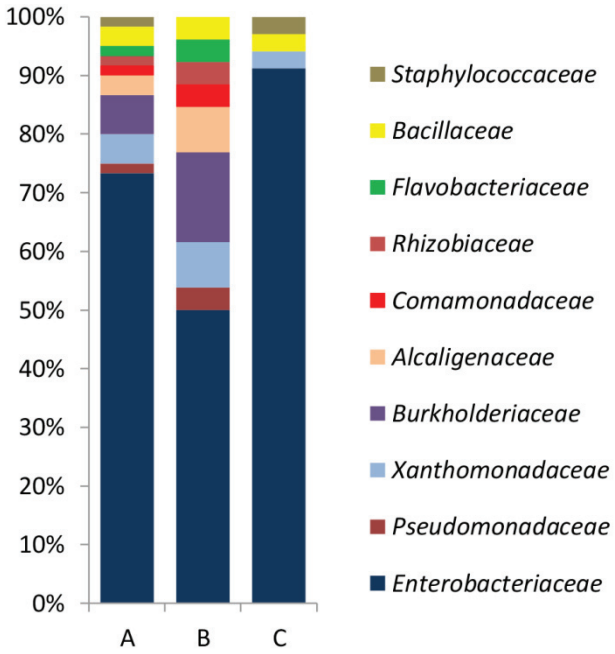

Figure 5.1. Microbial community composition, diversity and relative abundance of: total of bacteria carried by PWN (A), bacteria carried by PWN from *P. sylvestris* (B) and from *P. nigra* (C). The families: *Alcaligenaceae*, *Bacillaceae*, *Burkholderiaceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Flavobacteriaceae*, *Microbacteriaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, *Rhizobiaceae*, *Staphylococcaceae* and *Xanthomonadaceae*.

The bacterial isolates carried by *B. xylophilus* of the class *Alphaproteobacteria* belonged to the genus *Rhizobium* (1.7%). The isolates belonging to the class *Betaproteobacteria* were identified as strains of the families *Burkholderiaceae* (6.7%), *Alcaligenaceae* (3.3%)

and *Comamonadaceae* (1.7%). One strain was identified as belonging to the *Flavobacteriia*, genus *Chryseobacterium* (Figure 5.2).

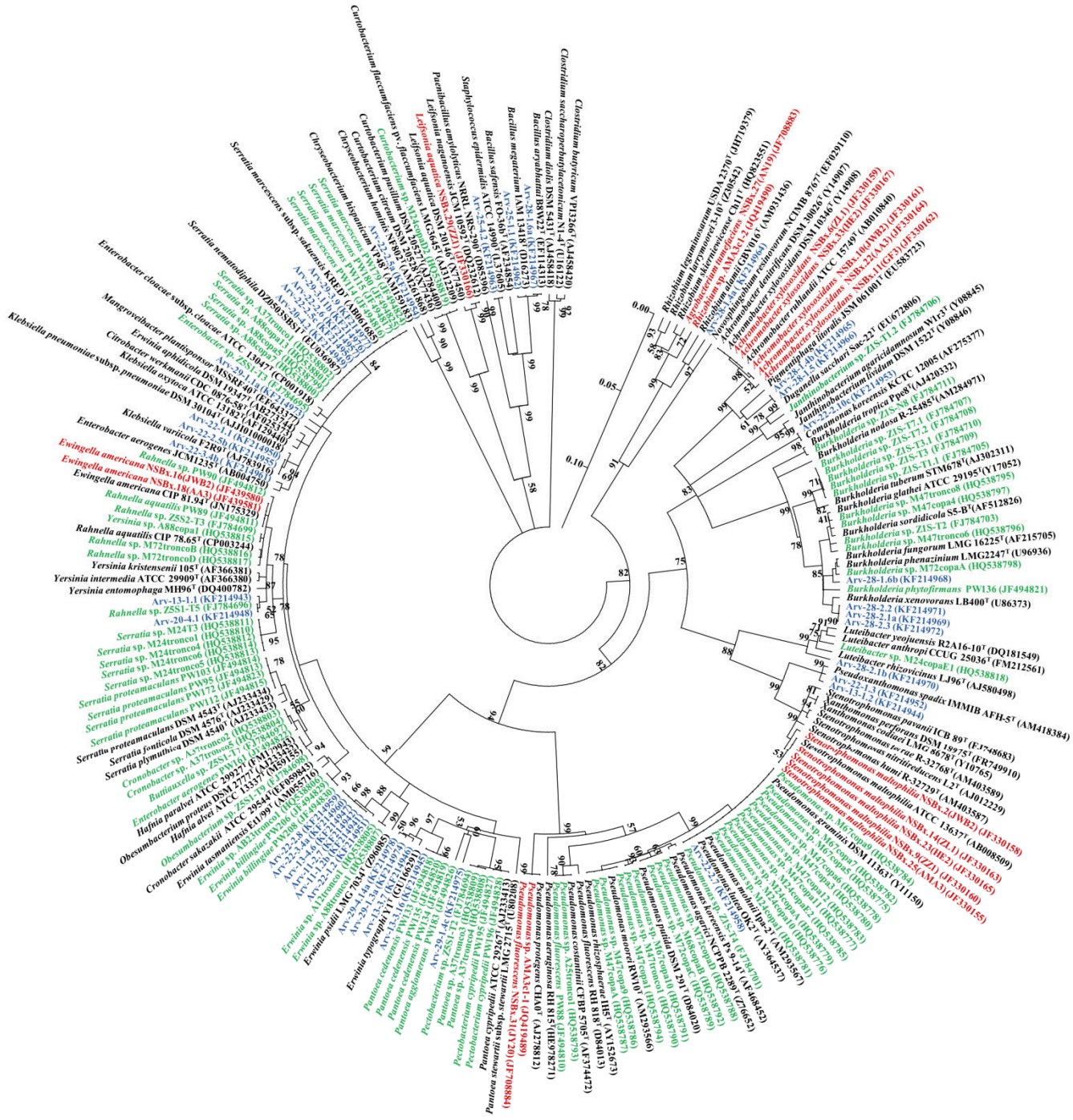

Figure 5.2. Phylogenetic analysis of bacterial 16S rRNA gene sequences of bacteria carried by PWN obtained from different countries (China - red, Portugal - green; USA - blue) and sequences available from NCBI. The circular tree was generated using a neighbor-joining analysis included in MEGA 5 software, partial deletion (95 %), linearized and rooted by *Clostridium* spp. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1,000 replications, values below 50% are not shown. Isolates characterized in this study are indicated in blue. Scale bar, 5 inferred nucleotide substitutions per 100 nucleotides.

The strains belonging to the class *Bacilli* from the families of *Bacillaceae* (3.3%) and *Staphylococcaceae* (1.7%). In this study, the class of *Gammaproteobacteria* comprised strains from the families *Enterobacteriaceae* (73.3%), *Pseudomonadaceae* (1.7%) and *Xanthomonadaceae* (5%). The family *Enterobacteriaceae* included strains from the genera *Serratia*, *Ewingella*, *Enterobacter*, *Klebsiella*, *Mangrovibacter* and *Erwinia* (Figure 5.2) and was the most abundant family in both *P. sylvestris* and *P. nigra* trees (Figure 5.1). The most abundant species in the *Enterobacteriaceae* were *Ewingella americana* and *Erwinia typographi* representing 50% of the diversity inside the family, followed by the species *S. marcescens* (21%) that was the second most abundant (Figure 5.2). The family *Pseudomonadaceae* was represented by six strains of the genus *Pseudomonas*. The class *Betaproteobacteria* was represented by strains of *Burkholderia xenovorans* (43%) and *B. phenazinium* (14%), two strains of the species *Pigmentiphaga litoralis* and one identified as *Comamonas koreensis*. The family *Xanthomonadaceae* was represented by two strains of genus *Pseudoxanthomonas* and one strain of genus *Dyella*. The class *Alphaproteobacteria* was represented by two strains, one of each genus *Novosphingobium* and *Rhizobium*. *Flavobacteriia* was represented by one strain of species *Chryseobacterium hominis*. Moreover, the Gram-positive bacteria isolates in this study were identified as belonging to the phylum *Firmicutes*. Bacteria inside *Firmicutes* were identified as *Bacillus megaterium* (66.7%) and *Staphylococcus epidermidis* (33.3%).

Comparing all the sampling areas, the microbial community carried by the nematodes was different for each sampling area. In the G area, only yeasts but no bacteria were detected associated with nematodes. The genus *Bacillus* was found in the other four sampling areas but just in nematodes from the pine *P. sylvestris*. The species *Pseudoxanthomonas spadix* was common to D and UNL sampling areas. Strains from the family *Enterobacteriaceae* were present in UNL, D and A areas. Although, strains from the genera *Pseudomonas* and *Burkholderia* were only found in UNL and W sampling areas, respectively.

5.4.3. Relationships between pine trees species, bacterial communities and environmental variables

In order to understand the relationships between pine tree species and bacterial communities carried by nematodes in different environmental conditions, the data obtained were explored by RDA (Figure 5.3).

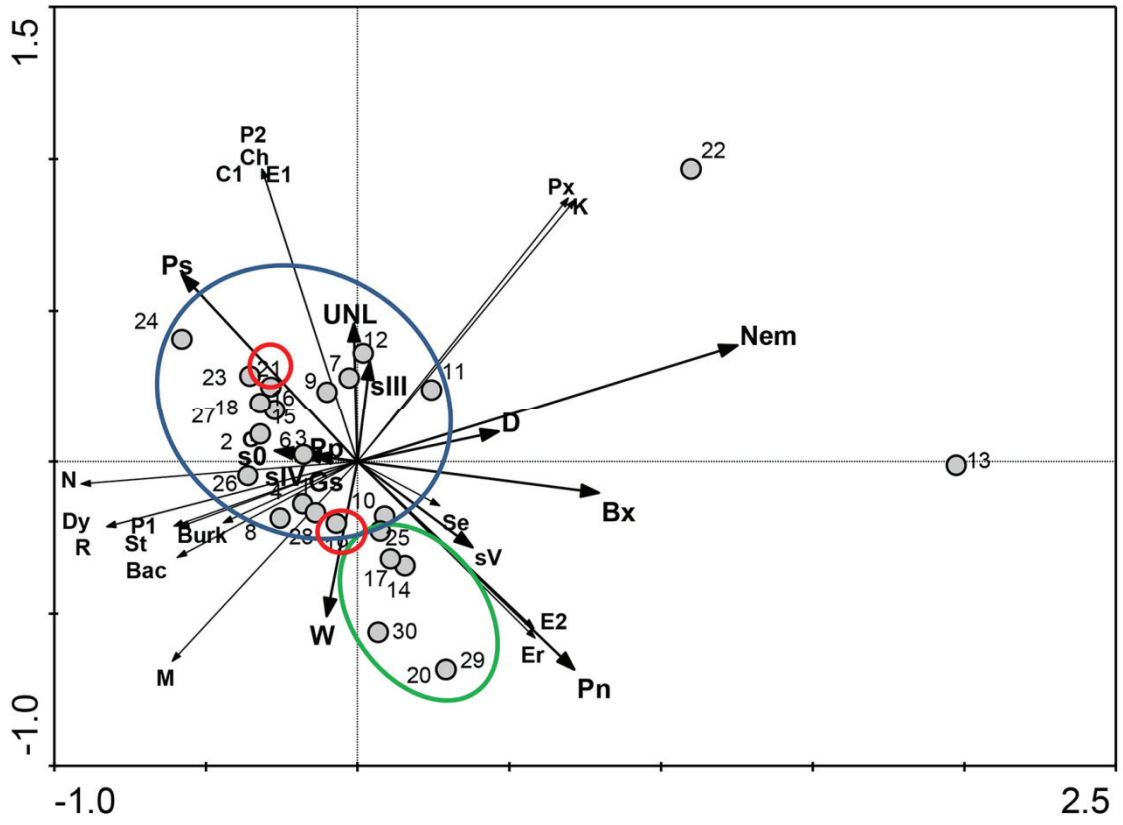

Figure 5.3. Relationships between pine trees species, bacterial communities and environmental variables. Redundancy analysis (RDA) performed with bacterial communities carried by PWN from five sampling sites. Circles highlight the closest relationship between *P. sylvestris* (blue), *P. nigra* (green) and *P. ponderosa* (red). The cumulative percentage of first and second axis explained 80.4 % of variance. The genera included in this analysis are: Bac - *Bacillus*, Burk - *Burkholderia*, C1 - *Comamonas*, Ch - *Chryseobacterium*, Dy - *Dyella*, E1 - *Enterobacter*, E2 - *Ewingella*, Er - *Erwinia*, K - *Klebsiella*, M - *Mangroveibacter*, P1 - *Pigmentiphaga*, P2 - *Pseudomonas*, Px - *Pseudoxanthomonas*, R - *Rhizobium*, Se - *Serratia*, St - *Staphylococcus*. The environmental variables are: sampling areas (G, Gs, W, D, UNL), pine tree species (Pn – P. nigra, Pp – *P. ponderosa*, Ps – *P. sylvestris*), presence of *B. xylophilus* (Bx) or other nematodes (Nem), and PWD symptom classes (s0, sIII, sIV, sV). The numbers are the pine trees sampled (Table 5.1).

The first and second axes explained 53.4 % and 80.4 % of cumulative variation, respectively. The first axis, representing the direction of maximum variation through the data, seems to separate the pine trees, based on the presence or absence of nematodes. The analysis revealed two main groups, one constituted by *P. sylvestris* trees and the other by *P. nigra* trees (Figure 5.3). However, the two pine trees of species *P. ponderosa* are included in group of *P. sylvestris* trees. Moreover, the pine trees

13 (*P. nigra*) and 22 (*P. sylvestris*) are outsiders, most probably due to the presence of other nematodes but they are separated depending on their *Pinus* species.

5.4.4. Phylogenetic relationship between strains carried by *B. xylophilus* from different countries

Next, we considered all the 16S rRNA sequences from bacterial strains carried by *B. xylophilus* from all the different countries with available data (USA, Portugal and China), with accession numbers mentioned in the literature and found in the NCBI database, a total of 136 sequences. Strains from Portugal were isolated using the same methodology as the one used in this study (Chapter 4; Proença *et al.*, 2010), or by using several selective (*Pseudomonas* complex medium) and non-selective media (Trypticase Soy Agar (TSA), Nutrient Agar (NA), Luria Agar (LA)) incubated at 28°C for 1 week (Vicente *et al.*, 2011b). The strains from China were isolated using NA or Nutrient Broth (NB) incubated for 2-3 days at 30°C (Wu *et al.*, 2013; Zhu *et al.*, 2012). The majority of the strains belonged to the class *Gammaproteobacteria*, others belonged to the class *Betaproteobacteria* and some strains to the class *Alphaproteobacteria*. Thirteen strains isolated from USA did not group with other isolates and belonged to the species *Mangroveibacter plantisponsor* (EF643377) (Arv-29-1.1a), *Klebsiella variicola* (AJ783916) (Arv-22-1.1, Arv-22-2.5b), *Erwinia typographi* (GU166291) (Arv-29-1.3a, Arv-29-4.14a, Arv-13-3.7), *Pigmentiphaga litoralis* (EU583723) (Arv-28-1.4b, Arv-28-1.5), *Comamonas koreensis* (AF275377) (Arv-22-2.10c), *Pseudoxanthomonas spadix* (AM418384) (Arv-13-1.2, Arv-22-1.3), *Chryseobacterium hominis* (AM261868) (Arv-22-2.5a), and *Bacillus megaterium* (D16273) (Arv-25-1.1, Arv-28-1.6a). The clade grouping strains from the genera *Ewingella* and *Rahnella* included strains from the 3 countries analyzed. Bacteria from the genera *Serratia* and *Pantoea* were both grouped into 2 subgroups and did not included strains from China. The genus *Pseudomonas* grouped strains from the 3 countries. Strains from the genus *Burkholderia* grouped strains from Portugal and USA. Most isolates from China belonged to the genus *Achromobacter* and grouped together with 2 strains of *Pigmentiphaga* from USA, both belonging to the *Alcaligenaceae*. Another 2 strains from USA belonged to *Pseudoxanthomonas* and grouped with the *Stenotrophomonas* strains from China.

5.5. Discussion

It is assumed that in Pine Wilt Disease, *B. xylophilus* is the sole causal agent but bacteria, according to recent findings (Zhao & Lin, 2005), may play a role in the disease progression, as bacteria from several genera have been found associated with the PWN.

Functional relationships have been suggested between *B. xylophilus* and bacteria (Jones *et al.*, 2008). Further, a genetic relationship between the two groups is supported by the presence of prokaryotic genes in the genome of nematodes from different families suggesting a co-evolutionary process with horizontal gene transfer (Kikuchi *et al.*, 2011; Scholl & Bird, 2011; Scholl *et al.*, 2003). Until now, different studies have demonstrated that PWN, obtained from infected trees from different countries, have an associated bacterial community (Chapter 4; Han *et al.*, 2003; Kawazu *et al.*, 1996a; Kwon *et al.*, 2010; Proença *et al.*, 2010; Vicente *et al.*, 2011b; Wu *et al.*, 2013; Zhao *et al.*, 2003; Zhu *et al.*, 2012). However, the comparison between the bacterial species associated with the nematodes from different countries is very difficult because the bacterial identification of most of the works were based on phenotypic characterization, using different methodologies, and not based on the 16S rRNA gene sequence of the strains. In the present work, all the bacterial strains carried by *B. xylophilus*, with public accession numbers in the NCBI database, were included for comparison of the bacterial communities. Only strains from USA, China and Portugal were found in the database and compared phylogenetically. Although, different methodologies were used for bacterial isolation all were based on non-selective media at similar incubation temperature that potentiating the isolation of mesophilic bacteria (Chapter 4; Proença *et al.*, 2010; Vicente *et al.*, 2011b; Wu *et al.*, 2013; Zhu *et al.*, 2012).

In the USA, as in Portugal, most of the bacterial strains carried by PWN belonged to the family *Enterobacteriacea* independent of the sampling area, and *Burkholderiaceae* were only found associated with nematodes from one area (Chapter 4; Proença *et al.*, 2010). Nevertheless, when considering the different parameters, the PWN-associated bacterial populations seemed to be related with the geographical area as well as pine tree species. Most of the bacterial genera carried by USA nematodes are strains described as plant associated organisms, either plant growth promoting or plant pathogens, some genera including species with opposite functions. Strains of *A. xylosoxidans* (family *Alcaligenaceae*) and from *S. maltophilia* (family *Xanthomonadaceae*)

are versatile, able to degrade aromatic molecules such as pyrene. Both species were isolated from *B. xylophilus* strains considered having moderate virulence (Ryan *et al.*, 2009; Wu *et al.*, 2013). *B. megaterium* also isolated in association with the nematodes, is a phosphate solubilizing bacterium, considered plant growth promoting (El-Hadad *et al.*, 2011). On the other hand, most of the strains from the genera *Pantoea* and *Erwinia* are phytopathogens. So, if PWNs are carrying bacteria from tree to tree (inside the insect vector) they could at the same time, introduce beneficial and harmful bacteria into the pine trees. However, recently studies showed that some strains from the genera *Serratia* and *Pseudomonas* have a nematicidal activity against *B. xylophilus* (Chapters 4, 6 and 7; Paiva *et al.*, 2013; Proença *et al.*, 2012a, b). Thus, a third role can be envisaged for the associated bacteria which is their negative activity against PWN.

The bacterial strains associated with PWN detected in the NCBI database were from China, Portugal and USA, and belonged to 25 different genera. However, only strains from the genus *Pseudomonas* were isolated from nematodes of each country. *Pseudomonas* is a very diverse group including species with very different functional characteristics. The strains from China were closely related to *P. fluorescens* and did not group with *Pseudomonas* strains isolated from Portugal or USA, which in turn were most related with the species *P. mohnii* and *P. lutea*. The *P. fluorescens* strain AMA31c1-1 (Zhu *et al.*, 2012) grouped with the *P. fluorescens* strain NSBx.31 (Wu *et al.*, 2013) from China. Han and coworkers (2003) suggested that *P. fluorescens* may be correlated with nematode virulence (Han *et al.*, 2003), but strain NSBx.31 (Wu *et al.*, 2013) had no visible effects on the virulence of PWN and strain AMA3c1-1 protected the plant against PWD (Zhu *et al.*, 2012).

Taking in consideration all variables, the pine trees were statistically grouped in two major groups which seem to be related with tree species (although, *P. ponderosa* grouped with *P. sylvestris*), the presence of some bacterial genera and the presence of other nematodes.

In conclusion, nematodes from the different countries are associated with bacteria of different species suggesting unspecific relationship. In addition, the diversity of the bacteria carried by the pinewood nematode seems to be dependent on the geographic area and the pine tree host species. The role of the different bacteria species inside the tree is probably independent of the presence of the nematode and only dependent on the bacteria's relationship with the tree.

5.6. Acknowledgements

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6

Nematicidal Bacteria Associated to Pinewood Nematode Produce Extracellular Proteases

Results published in:
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6.1. Abstract

Bacteria associated with the nematode *Bursaphelenchus xylophilus*, a pathogen of trees and the causal agent of pine wilt disease (PWD) may play a role in the disease. In order to evaluate their role (positive or negative to the tree), strains isolated from the track of nematodes from infected *Pinus pinaster* trees were screened, *in vitro,* for their nematicidal potential. The bacterial products, from strains more active in killing nematodes, were screened in order to identify and characterize the nematicidal agent.

Forty-seven strains were tested and, of these, 21 strains showed capacity to produce extracellular products with nematicidal activity. All *Burkholderia* strains were non-toxic. In contrast, all *Serratia* strains except one, exhibited high toxicity. Nematodes incubated with *Serratia* strains showed, by SEM observation, deposits of bacteria on the nematode cuticle. The most nematicidal strain, *Serratia* sp. A88copa13, produced proteases in the supernatant. The use of selective inhibitors revealed that a serine protease with 70 kDa, was the major responsible for the toxicity of the supernatant. This extracellular serine protease is different phylogenetically, in size and biochemically from previously described proteases. Nematicidal assays revealed differences in nematicidal activity of the proteases to different species of *Bursaphelenchus,* suggesting its usefulness in a primary screen of the nematodes.

This study offers the basis for further investigation of PWD and brings new insights on the role bacteria play in the defense of pine trees against *B. xylophilus*. Understanding all the factors involved is important in order to develop strategies to control *B. xylophilus* dispersion.

6.2. Introduction

A large number of species belonging to different phylogenetic groups such as viruses, bacteria, fungi, insects and some invertebrates have been found capable of invading or preying on nematodes (Siddiqui & Mahmood, 1996; Stirling, 1991). Nevertheless, chemical control is still the most commonly used method for managing nematode pests (Hague & Gowen, 1987).

Bursaphelenchus xylophilus, the pine wood nematode (PWN), is one of the most concerning species, since it is a pathogen of coniferous trees and the causal agent of the pine wilt disease (PWD). *B. xylophilus* is native to North America where it is considered to cause little damage to trees. However, in regions where it was introduced, as for example Japan, China or Europe, it is one of the most destructive pests of forest and landscape pines. Understanding the infection process, and all the factors involved, in order to develop strategies to control *B. xylophilus* dispersions has been an objective in several continents (reviewed in Jones *et al.* (Jones *et al.*, 2008)).

During PWD development, it was observed that cell death in the host tree seems to occur in advance to the increase of nematode population. In order to find a justification for this observation, some authors considered the presence of phytotoxins, including 8-hydroxycarbotanacetone and 10-hydroxyverbenone, identified in nematode-infested plants, responsible for cell death in the host (Oku *et al.*, 1980; Shaheen *et al.*, 1984).

More recently, a potential role for bacterial symbionts in the disease process has been anticipated. Nevertheless, there are no data from USA or Europe to support the idea that *B. xylophilus* interact in symbiosis with bacteria to cause the disease.

A series of studies have shown that bacteria from various genera could be isolated associated with *B. xylophilus* (Chapter 4; Han *et al.*, 2003; Higgins *et al.*, 1999; Proença *et al.*, 2010; Vicente *et al.*, 2012; Wu *et al.*, 2013). The presence of bacteria on the nematode surface has been described (Kusunoki, 1987), and a report on the observation of bacteria, by transmission electron microscopy, in the intestine of PWN was also performed (Yuan *et al.*, 2011). Lately, a Gram negative bacterium, *Stenotrophomonas maltophilia*, isolated from soil, was described (Huang *et al.*, 2009) to have nematotoxic activity against *B. xylophilus*, killing 65% of the nematodes after 24h of incubation. The analysis of the virulence factors revealed the presence of an extracellular serine protease of 28 kDa able to digest the nematodes cuticle. Among

the hydrolytic enzymes, serine proteases have recently been shown to be very important in the penetration and digestion of nematodes by nematode-trapping fungi (Ahman *et al.*, 2002; Lopez-Llorca *et al.*, 2002; Wang *et al.*, 2006; Yang *et al.*, 2005).

Bacteria associated with *B. xylophilus* and other nematodes isolated from *Pinus pinaster* trees with pine wilt disease (Chapter 4; Proença *et al.*, 2010) from recently nematode invaded areas (pine forest areas invaded in 2008), were diverse and belonged to different species according to the geographical area where the nematodes were isolated (Chapter 4; Proença *et al.*, 2010). *P. pinaster* from these studied areas had also a diverse endophytic microbial community (Chapter 2).

In order to evaluate the potential of these associated bacteria to produce negative effects on the nematodes, the isolates associated to the nematodes infecting *P. pinaster* from new invaded areas in Portugal (Chapter 4; Proença *et al.*, 2010) were screened for their potential in killing *B. xylophilus in vitro*. The bacterial strains, more active in killing nematodes, were selected and the bacterial extracellular products, produced during growth, were studied in order to determine their nematicidal activity.

6.3. Material and Methods

6.3.1. Bacterial isolation

Bacteria strains used in this work were isolated in Proença *et al*. (Chapter 4; Proença *et al.*, 2010). Briefly, *P. pinaster* trees were sampled in three different Portuguese areas affected by PWD: one located in southern Portugal, Setúbal District, between Alcácer do Sal and Grândola (Z) and two other areas in Central Portugal, Coimbra District, located in Malhada Velha, Arganil (M) and Avô, Oliveira do Hospital (A). The area between Alcácer do Sal and Grândola (Z) is affected with PWD since 1999 and the areas M and A are affected with PWD since 2008 (Mota *et al.*, 1999). The presence of nematodes was screened in all symptomatic trees. The bark and sapwood of infected trees were removed under sterile conditions, and the wood cut in ca. 2 cm chips. The wood pieces were placed in Petri dishes with R2A medium and incubated at 25°C, for three days. All bacterial colonies were isolated from the trails made by the nematodes on the medium. Isolated strains were preserved in LB medium with 15% glycerol at -80°C.

6.3.2. Screening and identification of bacterial strains with nematicidal activity

The bacterial strains, isolated under the conditions described above, were grown in Casamino Acids liquid medium (CAA) (Mossialos *et al.*, 2000) at 180 rpm, during 24h until the end of the exponential phase, at 26°C. The suspensions obtained were centrifuged (20 min, 4°C, 4000 rpm), in order to drastically reduce the number of cells, without removing any growth product (filtration with 0.22 µm filter showed to affect nematicidal activity in some strains). To test the nematicidal activity, 500 µl of each bacteria supernatant, with less than 0.06 $O.D._{600nm}$, were incubated with 75 (3 times 25) disinfected nematodes, for 48 hours at 26 °C. Nematodes were disinfected by sequential washes in sodium hypochlorite 0.1 % (one wash, 1 min at 4°C) and in 1 ml sterilized water (two washes, 3 min at 4°C), followed by centrifugation. Last water (100 µl) was inoculated on R2A for control of the disinfection efficiency.

The number of dead nematodes was assessed under the stereoscopic microscope. Nematodes were considered dead when linearized and not able to recover after being

transferred to water. All tests were performed in triplicate and repeated at least two separate times. The controls - nematodes in CAA and nematodes in water - were incubated under the same conditions.

The bacterial strains with a significant nematicidal activity in the above screening were selected and identified by 16S rRNA gene sequencing and comparison with sequences available in the EMBL/GenBank database, using BLAST network services, and with sequences in the EzTaxon identification service (Chun *et al.*, 2007).

6.3.3. Screening for extracellular proteases

To screen for the presence of proteases, all strains were grown in 50 ml of CAA at 26°C during 24h, centrifuged (20 min, 4°C, 4000 rpm) and the resulting supernatants were evaluated for the presence of proteins by SDS-PAGE using slab gels (1.5mm thick, 10% polyacrylamide) (Laemmli, 1970). The proteins were stained with Coomassie Blue G-250. Additionally, the proteolytic activity of the supernatants was evaluated by zymography using 12.5% polyacrilamide gels containing 0.2% copolymerized gelatin. In parallel for comparison, a native gel without gelatin was also performed. Each supernatant (25 µl) was mixed, without boiling, with sample buffer (dilution 1:1 with 125 mM Tris-HCl, pH 6.8, containing 4% SDS and 20% glycerol) and applied to the gel. After electrophoresis, zymogram gels were renatured twice with 0.25% Triton-X-100, washed in distilled water and incubated overnight in activation buffer (Tris HCl 50 mM pH 8.0). Gels were stained with Coomassie Blue and proteolysis in zymograms was detected as clear zones in a blue background. The weight of the proteins was estimated with an appropriate marker (Precision Plus Protein Standard) for native gel analysis (Bio-Rad).

Extracellular proteases of the strain A88copa13 were assessed from supernatants as described above. To purify the extracellular proteases with proteolytic activity, the purification process was initiated with a step of concentration of the collected supernatant trough Millipore Centricon® Centrifugal Filter Unit with a cut-off for 30,000 MW. The protein content was determined in the elute by Bradford assay (Bio-Rad Protein Assay, Bio-Rad). The proteins were purified by anionic exchange in FPLC separation. The crude protein extract was applied to a Hitrap Q column (1 ml) (Amersham, Switzerland) equilibrated in (buffer A) 50 mM Tris HCl (pH 8.0) buffer. The column elution was performed by a linear gradient 0-50% of (buffer B) 50 mM Tris

HCl (pH 8.0) with 1 M NaCl for 20 minutes at 1 ml/ min flow rate. The eluted proteins were detected at UV 280 nm. Fractions were collected and assayed for protease and nematicidal activities.

To assess the protease activity, 200 µl of each fraction and 2 µl of each of the 12 different substrates indicated in Table 6.1 were added to 50 mM Tris HCl, pH 8.0. All substrates were at 10 mM concentration.

Table 6.1: Proteolytic inhibition profile of the supernatant from *Serratia* strain A88copa13 with Alanine-AMC substrate.

Inhibitor	**% remaining activity**
No inhibitor	100
Pefabloc SC (1mM)	12
1,10 Phenanthroline (10mM)	13
Amastatin (10µM)	43
TPCK (0.1mM)	48
TLCK (0.1mM)	51
E-64 (10µM)	57
Bestatin (10µM)	57
Aprotinin (0.08mM)	59
A1-Antitrypsin (0.002mM)	59
chymostatin (0.1mM)	63
Pepstatin (1µM)	68
EDTA (10mM)	74
PMSF (1mM)	74
Mn^{2+} (1mM)	152
Mg^{2+} (1mM)	61
Ca^{2+} (1mM)	51
Zn^{2+} (1mM)	34

The release of AMC was measured for 20 minutes at 37°C, using a microplate fluorometer SpectraMAX-GeminiEM (Molecular Devices, Sunnyvale, USA), at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The fractions with the higher proteolytic activity were tested for nematicidal activity as described below. The proteolytic inhibition profile using Ala-AMC as substrate was determined by adding to the protease activity assay different inhibitors (Table 6.1).

6.3.4. MALDI-TOF identification of the proteases and gene amplification in strain A88copa13

The bands in native acrylamide gel, corresponding to proteins with proteolytic activity, were excised and placed into 1.5 mL microtubes, covered with water and sent to be sequenced by MALDI-TOF/TOF (MS / MS) at IPATIMUP, Porto, Portugal. Briefly, the samples were digested with trypsin, followed by the search of proteins from the mass spectrum of the peptides obtained (PMF). Peptide fragmentation spectra were obtained by peptide sequencing of MS / MS peaks with higher signal-noise. Peptides resulting from autolysis of trypsin were used as internal calibrants. Searches in databases of proteins and in the NCBInr Swiss-Prot/UniProtKB selections for taxonomic Bacteria and all entries were carried out.

The detection of the genes coding for the proteases in strain A88copa13 was done by PCR amplification with PCR reaction mix (50 μl) containing: reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3), 100 μM (each) deoxynucleoside triphosphates (Promega, Madison, Wisconsin, USA), 0.2 μM (each) primer and 1.5 U *Taq* polymerase (Sigma, St. Louis, Missouri, USA). For the Serralysin gene amplification the primers were 50KRT (5'-TTACACGATAAAGTCCGTGGCG-3') and 50KFT (5'-TGAGTGGAATCGAACCAATGCA-3'). Amplification of Serine protease gene was performed by three sets of primers 70KF1 (5'-GCATGYGTTATCYGTCGTTTC-3') and 70KR1 (5'-CAGTTCGCCCTTATTATAMGCT-3'), 70KF2 (5'-AGTTAAGCGTTTCCTCRAC-3') and 70KR2 (5'-GATGCTCGCCTCTTCGTTCAC-3'), 70KF3 (5'-GAAACGGCCAAACGCAGCGTG-3') and 70KR3 (5'-ATCGTYKCGATGRGGGCTGA-3'). The PCR was performed with 30 cycles: 1 min at 94°C, 1 min at 53°C, and 1 min 30 sec at 72°C. PCR products obtained were purified using the JET Quick PCR Purification Spin Kit (Genomed GmbH, Löhne, Germany) according to the manufacturer's instructions and sequenced.

6.3.5. Nematicidal activity of the proteases

The FPLC fractions, presenting higher protease activity, were tested for nematicidal activity against *B. xylophilus* (Bx-Portugal11AS) and other species of the genus *Bursaphelenchus*. The nematodes from the species *B. tusciae* (Bt-Italy), *B. mucronatus* (Bm-Portugal2) and *B. conicaudatus* (Bc-Japan) were used to evaluate the specificity of

the proteases against the different species of nematodes, using the methodology described above. Inhibitors for the different classes of proteins were added to the FPLC fractions with protease activity and incubated during 20 min. Each mixture was then incubated with 25 nematodes, in triplicate, under the conditions described above, and the nematodes mortality was evaluated. Controls were performed by incubating nematodes with the protease inhibitors used, in order to evaluate their toxicity under the same conditions: 1 mM Pefabloc SC (Sigma), 10 mM EDTA (Sigma) and 10 mM 1, 10-Phenantrolin (Sigma Aldrish).

6.3.6. Scanning Electron Microscopy observations

PWNs subjected and not subjected to nematicidal tests were prefixed in 2% formaldehyde plus 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h at 4°C. The nematodes were post-fixed with 2% osmium tetroxide for 30 min, and then dehydrated with serial ethanol treatments (30, 50, 70, 90, and 95%, and twice in 100% ethanol) for 30 min at each concentration. Then, the nematodes were dried by a critical point dryer (JCPD-5; JEOL, Tokyo) using liquid carbon dioxide, and sputter-coated with Au-Pd using a magnetron sputter (MSP-1S; Vacuum Device, Ibaraki, Japan). Scanning electron microscope (SEM) images were acquired using a VE-8800 (Keyence, Osaka, Japan).

6.3.7. Statistical Analysis

The significance of the differences, observed between the different treatments with *Serratia* sp. A88copa13, was evaluated with analysis of variance (ANOVA), using Tukey's Multiple Comparison Test, considering: 1) the amount of NaCl in the supernatant, 2) the different fractions obtained after FPLC of the supernatant, and 3) different species of nematodes. The statistical analysis was performed with GraphPadPrism v5.0 for windows, GraphPad software, San Diego, California, USA, www.graphpad.com. All differences were considered to be statistically significant for $P < 0.05$.

6.4. Results

6.4.1. Screening of the bacterial nematicidal activity

Forty-seven bacterial strains (Table 6.2) were evaluated for their ability to kill *B. xylophilus* nematodes. From these, 21 strains shown to have nematicidal activity (100% of nematodes dead after the incubation period). Five belonged to the species *Serratia plymuthica*, five to *Pseudomonas lutea*, three to *Serratia marcescens*, two to *Rhanella aquatilis* and one strain to each of the species *P. koreensis*, *P. constantinii*, *P. putida*, *P. moorei*, *Pantoea cypripedii* and *Curtobacterium pusillum* (Table 6.2). None of the *Burkholderia* strains had nematicidal activity (less than 30% of the nematodes killed during the assay). The lack of nematicidal activity was also observed in one strain of the genus *Yersinia* and in two *Erwinia* strains, and in one *P. putida* strain.

All strains showed ability to produce siderophores except 4 strains from three different genera (*Burkholderia*, *Pseudomonas* and *Serratia*), all with different nematicidal activities. Proteolitic activity in skim milk was detected in the supernatant of *P. constantinii*, *L. rizhinovicinus*, and of the 4 strains of the species *S. marcescens*, all able to kill more than 70% the nematodes during the assays. Only strains from *R. aquatillis* (one strain), *S. marcescens* (four strains) and *P. constantinii* (one strain) were able to degrade all the different lipids tested including Tween 80 (Table 6.2). All these strains were able to kill more than 70% of the nematodes in the nematicidal assay.

The observation by SEM of the result of bacterial activity on the nematodes showed bacteria cells accumulated on the nematode cuticle (Figure 6.1). The incubation of the nematodes with bacteria lead to the degradation of the nematodes, and only bacteria-covered empty cuticles were visible after 48h incubation.

Table 6.2. Evaluation of the nematicidal ability of the bacteria isolated associated with nematodes. Characterization of some catabolic properties and siderophores production that can be involved in the nematicidal activity. Lipase activity was tested in Tween 20 (1), Tween 40 (2), Tween 60 (3) and Tween 80 (4).

Families Classification	Species identification	Strain	16S rRNA gene Accession number (DDBJ/EMBL/GenBank)	Nematicidal activity (%)	Siderophores[a] (CAS)	Proteases[a] (skim milk)	Lipases[a] 1	2	3	4
Microbacteriaceae	*Curtobacterium pusillum*	**M24copaD2**	HQ538819	100	+	-	-	+	+	-
Xanthomonadaceae	*Luiteibacter rizhinovicinus*	**M24copaE1**	HQ538818	70-90	+	+	-	-	-	-
Enterobacteriaceae	*Cronobacter* sp.	**A37tronco2**	HQ538803	70-90	+	-	+	+	+	-
	Cronobacter sp.	**A37tronco5**	HQ538804	70-90	+	-	+	+	+	-
	Erwinia sp.	**B23tronco1**	HQ538806	70-90	+	-	-	+	-	-
	Erwinia sp.	**A12tronco1**	HQ538805	0-10	+	-	-	-	-	-
	Erwinia sp.	**A88tronco1**	HQ538807	0-10	+	-	-	-	-	-
	Pantoea sp.	**A37tronco4**	HQ538808	70-90	+	-	-	-	-	-
	P. cypripedii	**A37tronco3**	HQ538809	100	+	-	-	-	-	-
	Yersinia intermedia	**A88copa1**	HQ538815	30-50	+	-	-	+	+	-
	Rhanella aquatilis	**M72troncoB**	HQ538816	100	+	-	+	+	+	+
	R. aquatilis	**M72troncoD**	HQ538817	100	+	-	-	+	-	-
	Serratia plymuthica	**M24tronco1**	HQ538810	100	-	-	-	-	-	-
	S. plymuthica	**M24tronco3**	HQ538811	100	+	-	-	-	-	-
	S. plymuthica	**M24tronco4**	HQ538812	100	+	-	-	-	-	-
	S. plymuthica	**M24tronco5**	HQ538813	100	+	-	-	-	-	-
	S. plymuthica	**M24tronco6**	HQ538814	100	+	-	-	-	-	-
	S. marcescens	**A37tronco1**	HQ538802	70-90	+	+	+	+	+	+
	S. marcescens	**A88copa5**	HQ538799	100	+	+	+	+	+	+
	S.marcescens	**A88copa7**	HQ538800	100	+	+	+	+	+	+
	S.marcescens	**A88copa13**	HQ538801	100	+	+	+	+	+	+
Burkholderiaceae	*Burkholderia* sp.	**M47tronco8**	HQ538795	0-10	+	-	+	+	+	+
	Burkholderia sp.	**Z1S-T2**	FJ784703	0	-	-	+	+	+	-
	Burkholderia sp.	**Z1S-T3**	FJ784709	0-10	+	-	+	-	+	+
	Burkholderia glathei	**M47tronco6**	HQ538796	10-30	+	-	+	+	+	+
	B. fungorum	**M72copaA**	HQ538798	0	+	-	+	+	+	+
	B. sordidicola	**M47copa4**	HQ538797	10-30	+	-	+	+	+	+
Pseudomonadaceae	*Pseudomonas* sp.	**M47copa1**	HQ538794	70-90	+	-	-	+	+	-
	P. constantinii	**A25tronco1**	HQ538793	100	+	+	+	+	+	+
	P. koreensis	**M47copa9**	HQ538786	100	+	-	-	+	+	-
	P. koreensis	**M47copaA**	HQ538787	70-90	+	-	-	-	-	-
	P. gengeri	**M72copaC**	HQ538789	70-90	-	-	-	-	-	-
	P. lutea	**M24copa1**	HQ538781	70-90	+	-	+	-	-	-
	P. lutea	**M24copa10**	HQ538776	70-90	+	-	+	+	+	-
	P. lutea	**M47copa3**	HQ538780	100	+	-	+	-	-	-
	P. lutea	**M47copa6**	HQ538778	70-90	+	-	+	-	-	-
	P. lutea	**M47copa11**	HQ538777	100	+	-	+	-	+	-
	P. lutea	**M47copa12**	HQ538783	70-90	+	-	-	+	-	-
	P. lutea	**M67copa5**	HQ538775	100	+	-	+	-	-	-
	P. lutea	**M67copa7**	HQ538782	100	+	-	+	-	-	-
	P. lutea	**M67copa9**	HQ538784	70-90	+	-	+	-	-	-
	P. lutea	**M24copaA**	HQ538779	100	+	-	+	+	-	-
	P. lutea	**M24copaE2**	HQ538785	70-90	+	-	-	-	+	-
	P. moorei	**M47tronco1**	HQ538790	100	+	-	-	-	-	-
	P. putida	**M67copa10**	HQ538791	100	+	-	-	+	-	-
	P. putida	**M68copaB**	HQ538792	70-90	-	-	-	-	-	-
	P. putida	**M72copaD**	HQ538788	10-30	+	-	-	-	-	-

[a]Data obtained from Chapter 4; Proença *et al.* 2010.

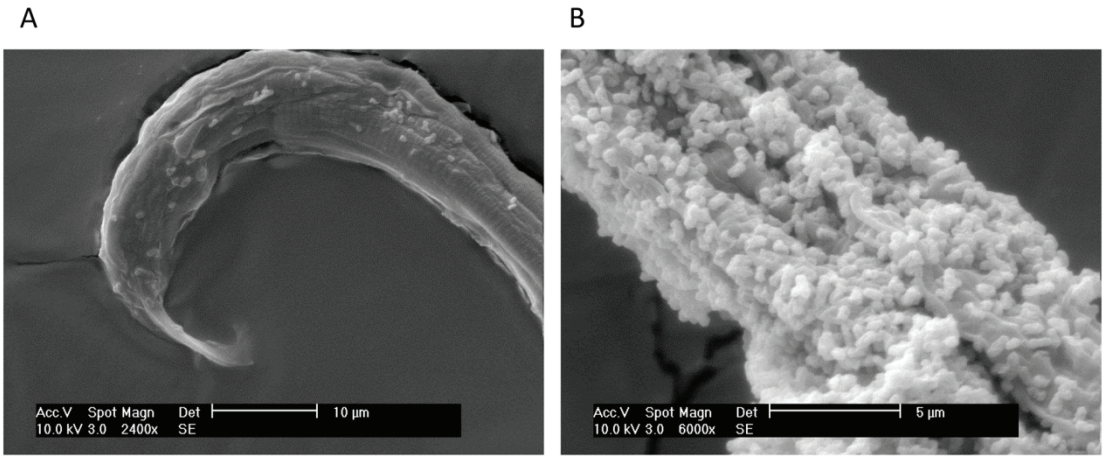

Figure 6.1. SEM images of deposits of *Serratia* strain A88copa13 on the cuticle of the nematode *Bursaphelenchus xylophilus*. Incubation after 24 h (A) and 48 h (B).

6.4.2. Proteases in the supernatants of nematicidal bacteria

The strains with higher nematicidal activity were selected to be screened for the presence of extracellular substances and proteins with proteolytic capacity. The strains A25tronco1, A37tronco1, A88copa5, A88copa7 and A88copa13 produced soluble extracellular proteins with proteolytic activity when evaluated by zymography with gelatin. Strain *S. marcescens* A88copa13 was selected for further work, because it produced the proteins with highest activity in the zymogram gel (Figure 6.2).

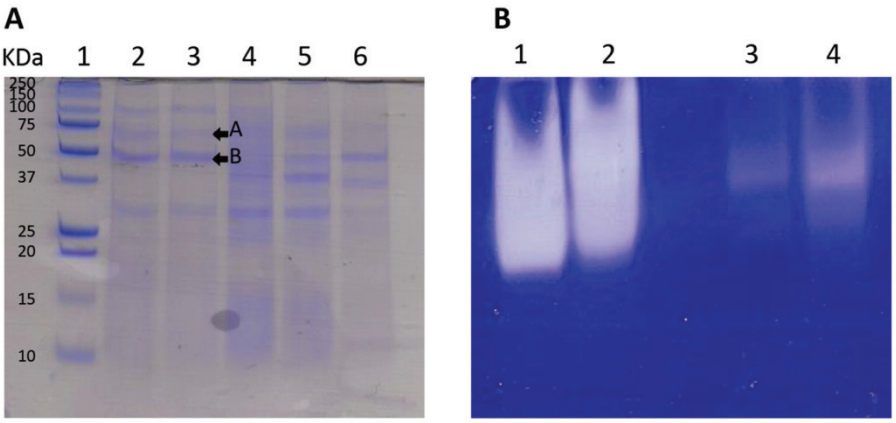

Figure 6.2. Proteins in the supernatants of nematicidal bacteria. (A) SDS-PAGE of extracellular proteins from *Serratia* strains A88copa13 (2 and 3) and A88copa5 (4, 5 and 6), molecular weight marker (1). Arrows indicate the band of 70 kDa (A-serine protease) and the band of 50 kDa (B-metalloproteinase serralysin) removed for sequencing by MALDI-TOF/TOF (MS / MS). (B) Zymogram using 12.5% polyacrilamide gel containing 0.2% copolymerized gelatin of extracellular proteins of *Serratia* strains A88copa13 (1 and 2) and A88copa5 (3 and 4) showing differences in protease activity.

6.4.3. Identification of extracellular proteins with proteolytic activity

The production of extracellular proteins by strain A88copa13, under the growth conditions used, was independent of the presence of nematodes (added dead to the growth medium) during strain growth (data not show). During growth of strain A88copa13, aliquots of the supernatant were removed at different times and the nematicidal activity was evaluated. The nematicidal activity of the strain's supernatant increased during the exponential phase of the strain's growth and was maximal when the strain achieved 2.4 $O.D._{610nm}$ (late exponential). Therefore, all assays were performed with aliquots removed from strain growth with 2.4 $O.D._{610nm.}$ The amount used to purify the protein fractions was standardized to 50 ml of supernatant. The protein content of the supernatant was 1.23±0.08 mg/ml, when quantified after centrifugation, and shown to be stable.

The toxicity assays were incubated for three days, and during this period the number of dead nematodes was counted each 24h. No increase in the number of dead nematodes was observed after 48h incubation. After this time, a brown color developed in the supernatant and the proteolytic activity ceased.

The presence and the number of proteins in the supernatant of the different nematicidal strains was evaluated by SDS-PAGE. The band from the supernatant of strain A88copa13 with 50 kDa (Figure 6.2) was identified by MS / MS, with 100% statistical significance (SwissProt database), as the extracellular metalloproteinase serralysin. The band with 70 kDa was identified, with 100% statistical significance (SwissProt database), as an extracellular serine protease. In strain A88copa13, the gene sequence obtained for the 70 kDa protease (JX667979) was 95% similar to the serine protease gene sequence of *S. marcescens* IFO-3046 (M13469). On the other hand, the 50 kDa band was coded by a gene (JX667978) which had 96% similarity with the *S. marcescens* strain ZhenJiang insecticidal protein gene (EU999787).

The comparative analysis of the amino acid sequence obtained from nucleotide translation of the 70 kDa protease using BlastX showed an high homology (96%, 87%, 50% and 49%, respectively) with the serine proteases of the subtilisin-type serine proteases family from two strains of *S. marcescens*, (P09489, P29805) and with a smaller serine protease (60 kDa) from *Xenorhabdus bovienii* (YP_003468240) and *X. nematophila* (YP_003712486). Similarly, the comparative analysis of the amino acid

sequence of the 50 kDa protease showed 99% homology with the zinc-dependent metalloproteinase, serralysin-like proteins from different strains of *S. marcescens* and *S. nematophila* (ACH90152, ABK55613, CAA39138 and ABZ81090).

6.4.4. Effect of protease inhibitors and metal ions on the enzyme activity

To confirm biochemically to which group the proteases in *Serratia* sp. A88copa13 belong, their activity was measured testing the supernatant in the presence of different protease inhibitors and protein substrates (Tables 6.1 and 6.3).

Table 6.3. Profile of proteolytic substrate specificity of the supernatant from strain *Serratia* sp. A88copa13

Substrate	**Activity*** *FU**/Min*
Ala-AMC (10mM)	314.70
Boc-Val-Pro-Arg-AMC (10mM)	216.42
BzArg-AMC (10mM)	97.56
Ala-Ala-Phe-AMC (10mM)	70.32
Phe-AMC (10mM)	41.42
Arg-AMC (10mM)	24.64
Met-AMC (10mM)	23.56
Lys-AMC (10mM)	22.79
Suc-Ala-Pro-Ala-AMC (10mM)	19.60
Leu-AMC (10mM)	13.30
Gly-Pro-AMC (10mM)	9.55
Suc-Leu-Leu-Val-Thr-AMC (10mM)	0.33

* 50 ml of supernatant
***FU* – Fluorescence Unity

The digestion of the fluorogenic substrates at pH 7.0 showed that Alanine-AMC and Boc-Val-Pro-Arg were the substrates that induced more activity (Table 6.3). These peptides were typical identified as metalloprotease (aminopeptidase) substrate and serine protease substrate, respectively.

To further characterize the extracellular proteases, enzymatic assays were performed with Alanine-AMC in the presence of class-specific protease inhibitors and divalent ions. In the presence of the substrate Alanine-AMC, the inhibitor Pefabloc SC (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; AEBSF), when added in concentrations of 1 mmol/L, almost completely abolished the proteolytic activity of the

supernatant, revealing a serine protease as a major active proteolytic enzymatic group (Table 6.1). The inhibitor 1,10-Phenanthroline, a chelating agent with high affinity to zinc, when added in concentrations of 10 mmol/L in the presence of Alanine-AMC as substrate, almost completely abolished the activity of the supernatant, also revealing an active metalloproteinase as a major proteolytic group, also.

In addition, with Alanine-AMC as substrate, Aprotinin showed to have an effect on the proteolytic activity of the supernatant and inhibited the proteolytic activity in 41%. EDTA is a metal ions chelator, which can chelate Ca^{2+} ions, and is recognized as an inhibitor of metalloproteinases. The addition of EDTA to the supernatant partially inhibited (26%) its proteolytic activity. Aspartic protease inhibitor, Pepstatin A, showed a moderate effect in the supernatant, with 32% of inhibition. Alkali metal ions showed a moderate effect on the enzymatic activity of the supernatant. However, Zn^{2+} inhibited the supernatant activity (66%) while Mn^{2+} increased it (152%).

The nematicidal activity of Pefabloc SC inhibitor was negative but EDTA and Phenantroline had a high nematicidal activity ($P<0.05$) (Figure 6.3).

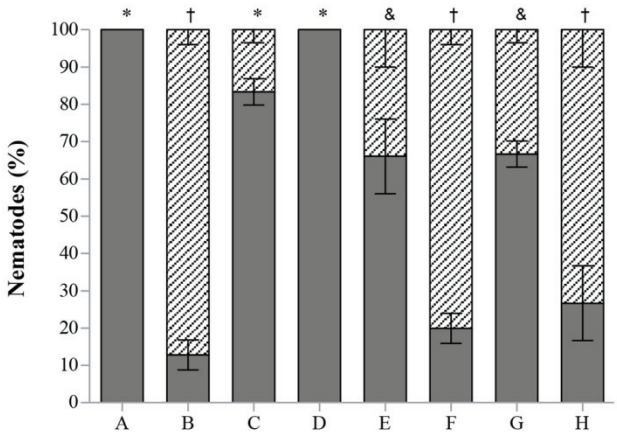

Figure 6.3. *Bursaphelenchus xylophilus* dead (stripes) and alive (grey) after the different *in vitro* treatments with *Serratia* strain A88copa13 (growth supernatant) and with proteases (FPLC fractions), after 24h incubation. The fractions with proteolytic activity contained 0.1M NaCl. A- H_2O; B- *Serratia* strain A88copa13; C- *Serratia* strain A88copa13 + Pefabloc SC; D- H_2O + Pefabloc SC; E- *Serratia* strain A88copa13 + EDTA; F- EDTA + H_2O; G- FPLC protein fraction 9; H- FPLC protein fraction 19. Different symbols (*, †, &) above the bars indicate significantly different percentages of mortality ($P < 0.05$) between treatments.

The high toxicity of the EDTA to the nematodes may suggest its action as a membrane permeabilizing agent, through the chelation of cations. When added to the supernatant,

the amount of cations available is higher than in sterilized distilled water, and the EDTA free to chelate cations from membranes will be less, lowering its toxicity. On the other hand, the possibility of the strain to produce EDTA degrading proteins to the supernatant cannot be overruled (Doronina *et al.*, 2010).

6.4.5. Nematicidal activity of FPLC fractions

Fractions of the supernatant separated by FPLC were tested for proteolytic activity and for their nematicidal activity (Figure 6.4A). A complete purification of the 2 proteases evaluated by SDS-PAGE and zymography was not achieved (Figure 6.4B).

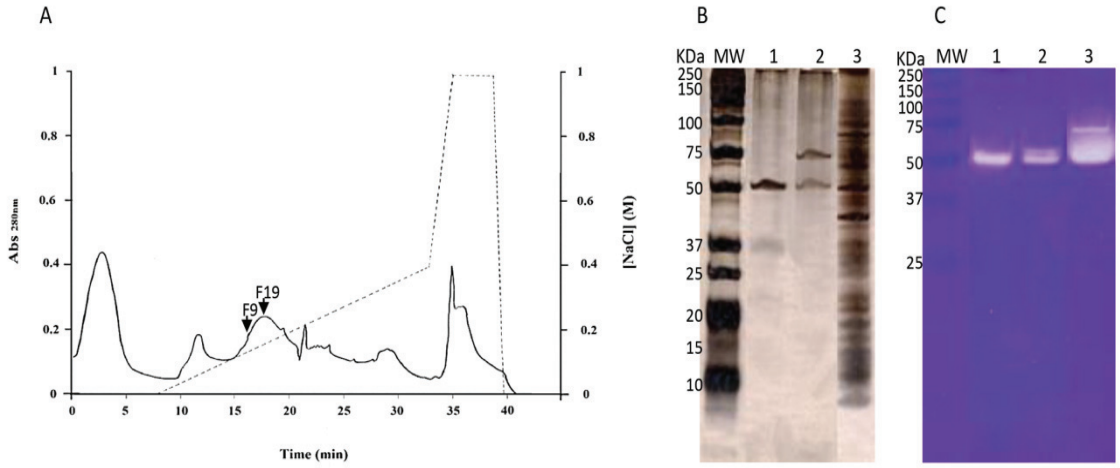

Figure 6.4. Purification and characterization of the proteases in the growth supernatant of strain *Serratia* A88copa13. (A) Anionic exchange chromatography on Hitrap Q column (1ml). Fractions 9 and 19 were eluted by using a linear gradient 0-50% of 50 mM Tris HCl (pH 8.0) with 1 M NaCl for 20 minutes at 1ml/ min flow rate. FPLC protein fraction 9 rich in metalloproteinase (1), FPLC protein fraction 19 rich in metalloproteinase and serine protease (2), A88copa13 supernatant (3), and molecular weight (MW) evaluated by SDS-PAGE silver stained (B) and by zymography using 12.5% polyacrilamide gel containing 0.2% copolymerized gelatin (C).

The FPLC fractions with proteolytic activity contained 0.1 M NaCl. Controls were performed in order to check the salinity effect on nematodes mortality. A control with the same salinity as the fractions tested (0.1 M NaCl), caused mortality similar to that observed with water (Figure 6.5). Statistical analysis showed that treatments with water (A, L, N, P), with proteins that did not bound to the FPLC column (B) and with buffer containing 0.1 M NaCl (G) did not induced nematodes mortality ($P>0.05$). The toxicity assays revealed that only two peaks showed nematicidal activity statistically comparable

to the whole extract (other treatments with $P<0.05$). The FPLC fractions 9 and 19 showed the highest toxicity, although with significant statistical differences ($P<0.05$) in consequence, most probably, of the presence of an additional serine protease in fraction 19 when compared with fraction 9. The activity measured by the hydrolysis of substrates and by the inhibition of the activity, determined in the presence of the specific inhibitors of each protein, demonstrated that the serine protease had the highest nematicidal activity. After 24 h incubation, 73% mortality was observed when nematodes were incubated with fraction 19 (data not shown). Extending incubation increased *B. xylophilus* mortality to 83% (Figure 6.5).

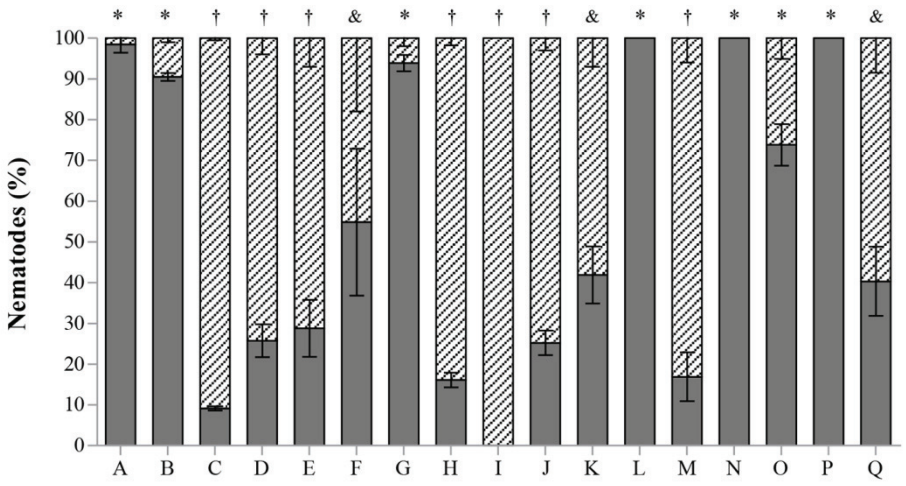

Figure 6.5: *Bursaphelenchus xylophilus* dead (stripes) and alive (grey) after the different *in vitro* treatments with *Serratia* strain A88copa13 (growth supernatant) and with proteases (FPLC fractions), after 48 h incubation. The fractions with proteolytic activity contained 0.1M NaCl. (A) H_2O + Bx-Portugal11AS; (B) FPLC fraction containing the proteins that did not bound to the column + Bx-Portugal11AS; (C) Tris-HCl + (1M) NaCl + Bx-Portugal11AS; (D) Tris-HCl + (0.7M) NaCl + Bx-Portugal11AS; (E) Tris-HCl + (0.5M) NaCl + Bx-Portugal11AS; (F) Tris-HCl + (0.3M) NaCl + Bx-Portugal11AS; (G) Tris-HCl + (0.1M) NaCl + Bx-Portugal11AS; (H) FPLC fraction 19 + Bx-Portugal11AS; (I) *Serratia* strain A88copa13 (growth supernatant); (J) FPLC fraction 19 (75%) diluted with H_2O; (K) FPLC fraction 19 (50%) diluted with H_2O; (L) H_2O + Bt-Italy; (M) FPLC fraction 19 + Bt-Italy; (N) H_2O + Bc-Japan; (O) FPLC fraction 19 + Bc-Japan; (P) H_2O + Bm-Portugal2; (Q) FPLC fraction 19 + Bm-Portugal2. Results from triplicate assays. Different symbols (*, †, &) above the bars indicate significantly different percentages of mortality ($P < 0.05$) between treatments.

Dilution of these fractions lead to a decrease in toxicity. The nematicidal activity of fraction 19 after 24 h incubation was also observed against nematodes isolates of the species *B. tusciae* (Bt-Italy) (81%), *B. conicaudatus* (Bc-Japan) (13%) and also *B. mucronatus*

(Bm-Portugal2) (10%). After 48h incubation, the nematicidal activity of this fraction increased significantly against Bm-Portugal2 and Bt-Italy ($P<0.05$) (Figure 6.5). Fraction 19 maintained the nematicidal activity against Bx-Portugal11AS until 72h of incubation but lost the activity when the incubation was prolonged (data not shown).

6.5. Discussion

Several studies have demonstrated that under natural conditions, *B. xylophilus* can carry bacteria (Chapter 4; Han *et al.*, 2003; Proença *et al.*, 2010; Zhao & Lin, 2005; Zhao *et al.*, 2003). Some authors are convinced that those bacteria might play an important role in PWN pathogenicity (Kong *et al.*, 2010; Zhao *et al.*, 2003) although the role of the bacteria carried by the nematode in PWD is not clear. The literature (reviewed in (Zhao & Li, 2008)) supports the idea that bacteria isolated from nematodes from PWD trees, interact in symbiosis with the nematode to cause the disease but also points to the fact that there is no data from USA or Europe to support that idea. Therefore, considering that, microorganisms can produce a wide range of secondary metabolites, the objective of this work was to investigate the bacteria carried by the PWN for their potential nematicidal properties.

The nematicidal ability was analyzed for 47 strains isolated associated to nematodes from trees with PWD. Only seven strains did not show toxicity against *B. xylophilus*, and *Burkholderia* was the only genus with all strains being non-toxic to the nematodes. Strains from the genus *Burkholderia* include well known human and plant pathogenic species (*B. gladioli*) but also some environmentally-important species such as *B. xenovorans* (chlororganic pesticides and polychlorinated biphenyls degrader) and some plant growth-promoting bacteria as *B. phytofirmans* (Estrada-De Los Santos *et al.*, 2001). However, the role *in vivo* of the strains of genus *Burkholderia* in *P. pinaster* with PWD was not evaluated. All strains of the genus *Pseudomonas,* except one strain of *P. putida,* showed toxicity against nematodes. The strains of the genus *Pseudomonas* are pointed by several authors as mutualistic with *B. xylophilus*, producing phytotoxins, and co-involved in PWD development (Guo *et al.*, 2007; Han *et al.*, 2003; Zhao & Lin, 2005). In this study, the nematicidal activity of the bacterial extracellular products was demonstrated but we cannot overrule their toxicity to plants. The genus *Serratia* included the strains most toxic to the nematodes: all except one strain were able to kill 100% of the nematodes in 24h incubation. The nematicidal activity of these strains is higher than the nematicidal activity of the previously described nematicidal strain *Stenotrophomonas maltophilia,* which was able to kill 65% of the nematodes after 24h incubation (Huang *et al.*, 2009). Strains from genus *Serratia* were also isolated associated with *B. xylophilus* JJ2 of *P. densiflora* collected in Korea (Kwon *et al.*, 2010). The strains of the genus *Serratia,* evaluated in this work, belonged to the species

S. plymuthica, considered non-pathogenic, and to the species *S. marcescens*, considered pathogenic to humans. *S. plymuthica* is a ubiquitous bacterium that has been preferentially recovered from rhizospheres all over the world, both as a free-living and as an endophytic organism. This species acts by antibiosis, by the production of lytic enzymes, by competition for nutrients and iron, by secretion of siderophores, and by induction of plant defense mechanisms (de Vleesschauwer & Höfte, 2007). The strains of *S. plymutica* tested in this work produced proteases and siderophores but not lipases, and toxicity against the PWN was demonstrated *in vitro*. The draft genome sequence of *Serratia* sp. M24T3 (*S. plymuthica* – like) showed multiple genes potentially involved in virulence and nematotoxity (Chapter 7; Proença *et al.*, 2012a) as the genes coding for colicin V and bacteriocin biosynthesis, as well as a set of genes typical for plant niche adaptation as, for example, acetoin (diacetyl) reductase that could be involved in plant protection against fungal and bacterial infections (Chapter 7; Proença *et al.*, 2012a). All strains from the species *S. marcescens*, differently from *S. plymutica* strains, produced lipases active on different polysorbates. This species was already reported as pathogenic to the pine sawyer beetle *Monochamus* (Shimazu, 2009).

In the process of enrichment of the proteases, aliquots were tested for proteolytic activity and for their nematicidal activity. The toxicity assays revealed that only two FPLC fractions (9 and 19) exhibited nematicidal activity comparable to the whole extract. The results from SDS-PAGE and enzymatic activity showed that these fractions included different concentrations of the two identified proteases (serine protease and serralysin). The use of selective inhibitors to serine proteases or to metalloproteinases demonstrated that the serine protease was the principal responsible for the toxicity on the PWN.

The production of serralysin-like proteases has been detected in several *Serratia* strains related to insects (Kim *et al.*, 2007). The metalloproteinase is also produced by the entomopathogens bacteria from the genus *Xenorhabdus* and by *Photorhabdus luminescens*, both symbiotic with their nematodes (Massaoud *et al.*, 2010). In our work, the selective inhibition of serralysin did not reduce the nematotoxicity of the supernatant of strain *Serratia* A88copa13, which suggests that serralysin is not the nematotoxic protease. This is in agreement to the fact that nematode-symbiotic entomopathogenic bacteria also produce serralysin-like proteases.

Hydrolytic enzymes produced by Gram positive bacteria have been identified to be involved in the degradation of nematode components (Cox *et al.*, 1981; Decraemer *et al.*, 2003; Huang *et al.*, 2005). Alkaline serine proteases have been reported to be produced by *Brevibacillus laterosporus* strain G4 (30 kDa designated BLG4) and from *Bacillus nematocida* (28 kDa) (Huang *et al.*, 2005; Qiuhong *et al.*, 2006) although both proteases were not the only virulence factor responsible for the nematicidal activities in these bacteria. Analysis of the results on nematode toxicity demonstrated that an extracellular serine protease was an important factor on strain A88copa13 virulence against the nematode *B. xylophilus*. The protease was also active against other nematodes of the genus *Bursaphelenchus*. However, considering the results after 24h, the nematicidal activity of the protease seems to be useful in the discrimination of the nematodes of the species *B. xylophilus* (100% of mortality with the supernatant) from the species of *B. mucronatus* (10% mortality). *B. mucronatus* is morphologically very similar to *B. xylophilus* and, although it infects the trees, it is not pathogenic to pine trees. This is according to previous studies showing that surface coat proteins, probably crucial in the nematode modulating or evading the host immune response, were different between virulent PWN and the avirulent *B. mucronatus* (reviewed in (Futai, 2013)). This serine protease is different, phylogenetically, in size and biochemically, from previously described proteases with nematotoxic activity but may play a similar role in infection against nematodes.

Therefore, this study offers a basis for further investigation of the mechanism of PWD caused by PWN and brings new insights on the role bacteria can play on defense against *B. xylophilus* in the maritime pine trees. The diversity of roles that can be assumed by the same bacterial species, as *Serratia marcescens*, goes from plant grow promoting to phytopathogen through the acquisition of genetic mobile elements or environmental stimuli (Ovcharenko *et al.*, 2010; Zhang *et al.*, 2005). Understanding all the involved factors is important in order to develop strategies to control *B. xylophilus* dispersions.

6.6. Acknowledgements

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7

Draft Genome Sequences of *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1 Carried by *Bursaphelenchus xylophilus* Isolated from *Pinus pinaster*

Results published in:

Proença, D. N., Espírito Santo, C., Grass, G. & Morais, P. V. (2012a). Draft genome sequence of *Serratia* sp. strain M24T3, isolated from pinewood disease nematode *Bursaphelenchus xylophilus*. *J Bacteriol* **194**, 3764–3764.

Proença, D. N., Espírito Santo, C., Grass, G. & Morais, P. V. (2012b). Draft genome sequence of *Pseudomonas* sp. strain M47T1, carried by *Bursaphelenchus xylophilus* isolated from *Pinus pinaster*. *J Bacteriol* **194**, 4789–4790.

7.1. Abstract

The draft genome sequences of *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1, carried by pinewood nematode, *Bursaphelenchus xylophilus*, the causative agent of Pine Wilt Disease, are presented. *Serratia* sp. M24T3 has been identified as a bionematocidal agent for *B. xylophilus in vitro* and multiple genes potentially involved in virulence and nematotoxity were identified. *Pseudomonas* sp. M47T1 harbors genes that potentially make this bacterium a plant growth-promoting bacteria and also genes putatively involved in nematotoxicity.

7.2. Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* is considered the causative agent of Pine Wilt Disease (PWD). Native to North-America, *B. xylophilus* was introduced to Japan and has spread into China, Korea and into Europe constituting a serious pest and a major pathogen of forest trees of *Pinus* species (Nickle *et al.*, 1981). Strains of the genus *Pseudomonas* were suggested to play a role in the disease (Zhao & Lin, 2005), but their specific functions and contributions to disease are still unclear. Isolations of *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1 were achieved from trails made on R2A solid media by *B. xylophylus* isolated from PWD-infected *Pinus pinaster* (Chapter 4; Proença *et al.*, 2010). Sequencing of the bacterial 16S rRNA gene was performed and the strains were tested for their ability to kill nematodes (Chapter 4; Proença *et al.*, 2010). Culture supernatants of both *Serratia* sp. strain M24T3 and *Pseudomonas* sp. strain M47T1 (classified as *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1 in (Chapter 4; Proença *et al.*, 2010)) killed *B. xylophilus* nematodes in vitro within 24 h of incubation (Chapter 6; Paiva *et al.*, 2013).

7.3. Material and Methods

7.3.1. Bacterial strains, media, growth conditions

Serratia sp. M24T3 and *Pseudomonas* sp. M47T1 were previously obtained carried by PWN (Chapter 4; Proença *et al.*, 2010) and grown in R2A broth medium at 30°C.

7.3.2. DNA extraction

The genomic DNA of both strains was extracted separately using QIAamp DNA Mini Kit (QIAGEN, USA) according to the manufacturer's instructions.

7.3.3. Genome sequencing and annotation

Reads were generated by 454 GS FLX sequencing (Margulies *et al.*, 2005) and raw data assembled using GS De Novo Assembler ("Newbler") Version 2.5.3. The assembled contigs were submitted to the RAST annotation server for subsystem classification and functional annotation (Aziz *et al.*, 2008). Coding sequences (CDSs) were assigned using BLASTp with KEGG Orthology (KO). The G+C content was calculated using an in-house Perl script. The NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) was employed for gene annotation in preparation for submission to GenBank (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html).

7.3.4. Nucleotide sequence accession numbers

The Whole Genome Shotgun projects of *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1 have been deposited at DDBJ/EMBL/GenBank under the accession AJHJ00000000 and AJWX00000000, respectively. The versions described here are the first versions, AJHJ01000000 and AJWX01000000, respectively. Both genome project data are also available at GenBank under the genome Bioproject IDs PRJNA104949 and PRJNA162465, respectively.

7.4. Results and Discussion

7.4.1. Genome Announcement of *Serratia* sp. M24T3

The draft genome sequence of *Serratia* sp. strain M24Tt3 comprises 5,257,417 bases, representing a 53-fold coverage of the genome. The assembled genome consists of 97 large contigs with more than 500 bp (N_{50} contig size was 132,002 bp). The G+C content was 49.03 %. The genome encodes 4,858 putative coding sequences (CDSs). The draft genome sequence contains eight ribosomal RNAs and sixty-three tRNAs loci. For the CDSs, 84 % corresponding to 4,030 proteins could be assigned to Cluster of Orthologous Groups (COG) families (Tatusov *et al.*, 2000). Comparison with genome sequences available at RAST showed that 30 of the closest strains belong to eight genera (*Citrobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella* and *Yersinia*).

Serratia sp. strain M24T3 genome carries multiple genes potentially involved in nematotoxic activity such as Colicin V and Bacteriocin biosynthesis, the *yafQ* gene encoding for a endoribonuclease (YafQ toxin) and a gene that contains a bacterial RTX (repeats in the structural toxins) toxin-activating protein-C signature.

The phylogenetic closest relative *S. plymuthica* is able to protect against plant pathogenic *Agrobacterium* (Dandurishvili *et al.*, 2011) and induced systemic resistance against various rice pathogens (de Vleesschauwer *et al.*, 2009). *Serratia* sp. strain M24T3 has a set of genes typical for plant niche adaptation like nitrogen regulatory protein P-II (ammonia assimilation), putative polysaccharide deacetylase (putatively involved in nodulation) and acetoin (diacetyl) reductase (plant protection against fungal and bacterial infections). Additionally it is tempting to speculate that *Serratia* sp. strain M24T3 may also be used as bionematocidal agent against certain nematodes.

7.4.2. Genome Announcement of *Pseudomonas* sp. M47T1

The draft genome sequence of *Pseudomonas* sp. strain M47T1 comprises 6,311,977 bases, representing a 49-fold coverage of the genome. The assembled genome consists of 72 large contigs with more than 500 bp (N_{50} contig size was 171,047 bp). The G+C content was 61.80 %. The genome encodes 5,678 putative coding sequences (CDSs). The draft genome sequence contains six ribosomal RNAs and sixty-nine tRNAs loci. For the CDSs, 82.4 % corresponding to 4,679 proteins could be assigned to Cluster of Orthologous Groups (COG) families (Tatusov *et al.*, 2000). Comparison with genome sequences available at RAST showed that 30 of the closest strains belong to nine genera (*Azoarcus*, *Azotobacter*, *Cellvibrio*, *Chromohalobacter*, *Hahella*, *Marinobacter*, *Marinomonas* and *Shewanella*) outside the pseudomonads.

The genome of *Pseudomonas* sp. strain M47T1 carries multiple genes potentially involved in nematotoxic activity such as Bacteriocin and Colicin V biosynthesis, the *YdhE/NorM* gene encoding for a homolog of a multidrug and toxin extrusion (MATE) family efflux pump, three genes coding for Rhs family protein that may be involved in adhesion or virulence (Kung *et al.*, 2012; Pradhan *et al.*, 2012) and a gene encoding a calcium-binding protein from the bacterial RTX toxins (repeats in toxins) family (Zhang *et al.*, 2012b).

Moreover, strain M47T1 also contains genes that are potentially involved in promoting plant growth, such as genes encoding for ferric siderophores and for phenazine biosynthesis (Compant *et al.*, 2005). This strain has also a set of genes typical for plant niche adaptation like the NodD that regulates the nodulation genes, nitrogen regulatory protein P-II (ammonia assimilation) and acetoin (diacetyl) reductase for plant protection against fungal and bacterial infections. In short, the genomic information suggests a positive interaction with the plant but a negative interaction with the PWN worm. Thus, *Pseudomonas* sp. strain M47T1 may be a good candidate to be developed as a bionematocidal agent against an important pest of conifers.

7.5. Acknowledgments

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8

Colonization of *Arabidopsis thaliana* by Nematicidal *Serratia* sp. M24T3 and its Effects on Plant Development

Results submitted in:

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8.1. Abstract

Serratia sp. M24T3 was found to be associated with *Bursaphelenchus xylophilus,* the pinewood nematode from pine wilt disease and showed nematicidal activity against *B. xylophilus*. The role of these bacterial strains inside pine trees remains unclear. This study aimed to evaluate the colonization process in *Arabidopsis thaliana* by *Serratia* sp. M24T3, in order to predict it as a competent endophyte, and to identify its genes potentially involved in plant growth promotion. *A. thaliana* seedlings were inoculated *in vitro* with *Serratia* sp. M24T3 carrying the *gfp*::*gusA* plasmid pHRGFPGUS (10^6 CFU.g^{-1}). Confocal laser scanning microscopy confirmed the presence of *gfp*-tagged M24T3 inside root tissue, intercellular spaces, inside plant cells and vascular zones. The presence of M24T3 decreased plant root elongation but increased the number of lateral roots, an effect probably related with strain production of IAA demonstrated *in vitro*. Strain M24T3 showed potential to be a plant growth-promoting bacterium, since it produced siderophores, solubilized phosphate and zinc oxide. Additionally, it has an ACC deaminase gene most probably involved in lowering plant ethylene levels. Results suggest strain M24T3 to be able to colonize other plants besides pine trees and to work as plant growth-promoting bacterium.

8.2. Introduction

Agrochemicals are used to protect plants against invasive pests (pesticides) as well as for plant improvement in yield (fertilizers). The massive use of chemicals resulted in tremendous impacts in farmers' economy and to the environment (Compant *et al.*, 2005). Besides chemical compounds, use of plant growth-promoting bacteria (PGPB) have been suggested as an alternative to the classical strategies (Compant *et al.*, 2010).

PGPB constitute an inhomogeneous group of microorganisms found in soil, rhizosphere or inside plant tissues (i.e., endophytes). PGPB provide beneficial functions for the plant host such as those involved in: i) production of phytohormones that support plant growth – auxins such as indole acetic acid (IAA), ethylene, gibberellins, cytokinins or abscisic acid; ii) production of enzymes, including bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase which has been reported to reduce the plant ethylene levels by degrade of its precursor (ACC) into α-ketobutyrate and ammonia; iii) phosphate and zinc solubilization, iron chelation by siderophores, nitrogen fixation and nitric oxide production; and iv) plant protection against pathogenic organisms by producing antagonistic compounds or by induced systemic resistance (ISR) (Gamalero & Glick, 2011; Glick *et al.*, 2007; Hardoim *et al.*, 2008; Reinhold-Hurek & Hurek, 2011; Ryan *et al.*, 2008; Stoltzfus *et al.*, 1997). On the other hand, PGPB need to cope with inside tree environment. Reactive oxygen species (ROS) and also nitric oxide (NO) are known as part of the innate immunity system of plants (Apel & Hirt, 2004; Oliveira *et al.*, 2009; Zeidler *et al.*, 2004). Therefore, on way of plant colonization, microbes need to have some antioxidant enzymes, for example superoxide dismutase (SOD) and catalase (Hartmann *et al.*, 2008). A variety of bacterial species of the genera *Azospirillum*, *Burkholderia*, *Gluconacetobacter*, *Herbaspirillum*, (Baldani *et al.*, 2002), *Rhizobium*, *Bradyrhizobium* (Rouws *et al.*, 2013), *Bacillus* (Fan *et al.*, 2011), or *Serratia* (Compant *et al.*, 2005) have been recognized to play a role as PGPB.

Recently, *Serratia* sp. M24T3 was found to be associated with the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease (Chapter 4; Proença *et al.*, 2010). The role of these bacterial strains inside pine trees remains unclear, and a positive role (nematotoxic activity against *B. xylophilus*) or a negative role (mutualistic relation with the nematode) has been suggested (Chapter 6, Paiva *et al.*, 2013; Zhao & Lin, 2005). Furthermore, the 16S rRNA gene sequence of this species

was also found as part of the natural endophytic community of *Pinus pinaster* (Chapter 1). Finally, strain M24T3 is able to kill the nematode (Chapters 6 and 7, Paiva *et al.*, 2013; Proença *et al.*, 2012a) acting as a bionematicidal agent *in vitro*.

In order to confirm that strain M24T3, isolated from PWN, is a competent endophyte with plant growth-promoting properties, the main goal of this study was to evaluate the colonization capability in the plant model *A. thaliana* by nematicidal *Serratia* sp. M24T3. Biochemical characterizations were performed, as well as a genomic survey for genes usually recognized as involved in plant growth promotion.

8.3. Material and Methods

8.3.1. Bacterial strains, plasmids, media, growth conditions and transformation

Serratia sp. M24T3 previously isolated from PWN (Chapter 4; Proença *et al.*, 2010) was grown in LB medium at 30°C. *Escherichia coli* S17-1 was used as a control for plant colonization. For strain M24T3 harboring plasmid pHRGFPGUS (*gfp*::*gusA*, $Amp^R Kan^R$) (Ramos *et al.*, 2002) kanamycin (50 $\mu g.ml^{-1}$) was added.

Electroporation of *Serratia* sp. M24T3 with plasmid pHRGFPGUS was performed using a Gene Pulser Xcell™ (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions for *E. coli* (2.5 kV, 25 µF, 200 ohm, 5.2ms). Fresh culture cells from strain M24T3 (OD_{600}=0.6 ≈ 10^6 $cells.ml^{-1}$) were used to prepare electrocompetent cells (Ausubel *et al.*, 1987) and all centrifugation steps were performed at 7,000 g during 25 min at 0°C. Two microliters of plasmid pHRGFPGUS were used for transformation. The cells were harvested and then resuspended in 1 ml of SOC medium and incubated 1h at 30°C. The cell suspensions were plated onto the LB agar with kanamycin.

8.3.2. Production of IAA, siderophores and proteases

The ability of strain M23T3 to produce IAA was measured based on colorimetric method (Glickmann & Dessaux, 1995; Patten & Glick, 2002) with modifications. Strain M24T3 (transformed and wild type) was grown in LB broth, either in the absence or presence of 500 $\mu g.ml^{-1}$ L-tryptophan at 30°C up to a final OD_{600}=0.6. One milliliter of each culture was centrifuged and 100 µl supernatant was mixed with 150 µl Salkowski's reagent (Gordon & Weber, 1951), incubated for 30 min at room temperature, and absorbance was measured at 540 nm. A standard plot was performed from 5 to 50 µg.ml-1 pure IAA (Sigma) in order to calculate the concentration of each sample. Assays were performed in triplicate. Protein quantification was performed in order to normalize the concentration of the production of IAA. In order to do it, the pellet was resuspended in 1 ml 0.8 % NaCl, 100 µl were removed and 100 µl 1M NaOH added, mixed and incubated for 5 min at 100°C. Twenty µl were added to 180 µl of Bradford reagent (Bio-Rad), incubated 10

min at room temperature in the dark and absorbance measured at 595 nm. Assays were performed in triplicate.

The capability to produce siderophores and proteases of strain M24T3 was previously evaluated (Chapter 4; Proença *et al.*, 2010) and revisited in this study.

8.3.3. Phosphate and zinc solubilization

The ability for phosphate and zinc solubilization of the strain M24T3 was determined on Pikovskaya's agar plates supplemented with 0.5 % $Ca_3(PO_4)_2$ and 0.12 % ZnO as inorganic P and Zn sources, respectively (Pikovskaya, 1948). The plates were incubated at 30°C up to 7 days and a transparent halos around the zone of growth were considered positive for both tests.

8.3.4. Nitrogenase, cellulolytic, chitinolytic, catalase and SOD activities

Strain M24T3 was screened to form pellicles in semi-solid R2A media without nitrogen sources in order to evaluate nitrogen fixation (Döbereiner *et al.*, 1995; Weber *et al.*, 2001). Strain M24T3 was screened for nitrogenase activity by the acetylene reduction assay (ARA) and for the presence of the *nifH* gene by PCR amplification according to previously work (Rouws *et al.*, 2013). Detection of cellulase activity was performed on R2A agar plates containing 1 % (w/v) carboxymethyl cellulose (CMC) and evaluation test was performed according to a previous work (Carder, 1986). The ability to hydrolyze chitin at concentration of 1.0 % (w/v) on R2A agar, after incubation at 30 °C up to 5 days, was determined according to previously work (Tindall *et al.*, 2007). The catalase and SOD activities were determined after 24 h of incubation on R2A agar as previously described (Smibert & Krieg, 1994).

8.3.5. *In silico* methods for plant growth-promoting properties

The genome of *Serratia* sp. M24T3 was previously published (Chapter 7; Proença *et al.*, 2012a) and it is available in DDBJ/EMBL/GenBank under accession no. AJHJ00000000. Some genes encoding for proteins that are recognized as important for plant colonization processes as well as for plant growth promotion were queried in the genome using the RAST server tools (Aziz *et al.*, 2008) as well as BLAST (Altschul *et*

al., 1997): 1-aminocyclopropane-1-carboxylate (ACC) deaminase; cellulase; pectinase; superoxide dismutase; catalase; chitinase. The *nif* genes and genes involved in ammonia, IAA, acetoin and 2,3-butanediol biosynthesis were also queried.

8.3.6. Phylogenetic analysis of ACC deaminase sequences

Sequence of ACC deaminase from *Serratia* sp. M24T3 was compared with sequences available in the EMBL/GenBank database using BLAST network services in order to reveal its phylogenetic relationship with the other ACC from the genus *Serratia* (Altschul *et al.*, 1997). Sequences were aligned with MEGA 5.1 software (Tamura *et al.*, 2011). Phylogenetic dendrograms were constructed by maximum likelihood method and Poisson correction as substitution model included in MEGA 5.1 software. Bootstrap analysis with 1,000 replicates was used to evaluate the robustness of the phylogeny.

8.3.7. Plant growth conditions for *A. thaliana* and bacterial inoculations

A. thaliana seeds, of genotypes Columbia or its derivative *DR5*::GUS, were disinfected in sterile microtubes by adding 500 µl (ethanol 70 % and Tween 0.05 %), and incubating for five minutes. The supernatant was discarded, 500 µl of 100 % ethanol, added and incubated by five minutes. The ethanol was discarded and evaporated. The disinfected seeds were seeded on half concentrated MS medium, supplemented with 1 ml/l of vitamins (mg/ml: 1.0 myo-inositol, 0.1 thiamine hydrochloride, 0.5 pyridoxine hydrochloride, 0.5 nicotinic acid, 2 glycine), 1.5 % glucose and 0.8 % agar (Murashige & Skoog, 1962). The plates were incubated at 4°C for three days to enhance germination.

The bacterial inoculations were performed with exponential growth phase cultures of OD_{600}=0.6. The bacterial suspensions for plant inoculations consisted in 1 ml of bacterial growth washed with MS medium ½ concentrated with vitamins and glucose but without agar (see above), pelleted (1 min, 13,000 rpm) and resuspended in 1 ml of ½ concentrated MS medium. In order to test promotion of seed germination, five seeds of *A. thaliana* were inoculated after disinfection together with 500 µl suspension of M24T3. Controls were performed using non-inoculated medium and *E. coli* S17 suspension. All the tests were performed in triplicate.

For root elongation assays, seeds were spread in a single line on MS agar plates (see above). 50 µl suspensions of strain M24T3 transformed with plasmid pHRGFPGUS and wild type, or bacterial supernatants were added separately in the center of different plates, on sterile filter paper (1 cm diameter) at two cm distance from the seeds line. Plates were incubated vertically and plants were grown at 24°C under a 16/8 h light-dark cycle.

Colonization assays were performed after germination of plantlets at 24°C for 7 days. Plantlets were transferred under sterile conditions, after germination on MS agar plates, into wells of 12-well plates. Each well contained 500 µl of bacterial suspensions (M24T3, transformed with plasmid pHRGFPGUS, wild type, or *E. coli* S17 transformed with plasmid pHRGFPGUS) and 5 plantlets. MS medium without bacteria was used as negative control. Plantlets were grown at 24°C under a 16/8 h light-dark cycle. All the tests were performed in triplicate. After 7 days of inoculation, *A. thaliana* plantlets were collected for microscopy analysis.

8.3.8. Light microscopy and confocal laser scanning microscopy (CLSM)

Seven days after inoculation in liquid MS medium *A. thaliana* plantlets uninoculated and inoculated with bacteria were removed from the 12-multiwell plate, washed with sterile water and mounted on bridge slide with 10% (v/v) glycerol. *A. thaliana* plantlets were observed by bright field microscopy under a Zeiss Axioplan microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Then, in order to observe colonization of *A. thaliana* by bacteria, plantlets were observed in a LEICA TCS-SP5 AOBS confocal system with 40 x lens and Leica Application Suite – Advanced Fluorescence Lite software (release 2.4.1). GFP-tagged bacterial cells were excited with the 488 nm Argon laser line (25% power). Chlorophylls from *A. thaliana* cells were excited with the 405 nm Diode laser line (20% power). All images were collected in a z-series with 15 optical sections, step size 2 µm and 52 µm of average thickness.

8.4. Results

8.4.1. *In vitro* PGPB properties of strain M24T3

Serratia sp. M24T3 showed positive result for phosphate and zinc solubilization, cellulolytic activity by CMC degradation test, produced siderophores but did not had proteolytic activity (Proença *et al.*, 2010). Strain M24T3 showed also chitinolytic activity by chitin degradation test as well as the enzymes catalase and SOD.

The production of IAA was similar by both *Serratia* sp. M24T3 wild type and transformed with plasmid pHRGFPGUS. The concentration obtained of IAA by strain M24T3 grown in LB was 4.48 µg/ml and 9.78 µg/ml, unsupplemented and supplemented with L-tryptophan, respectively. However, these concentrations when normalized by the total concentration of protein, the concentration was similar between unsupplemented and supplemented with L-tryptophan, i.e., the concentration was 0.035 $\mu g_{(IAA)}/(\mu g_{(protein)}/.ml)$ and 0.036 $\mu g_{(IAA)}/(\mu g_{(protein)}/.ml)$, respectively.

The *Serratia* sp. M24T3 was able to form pellicle in semi-solid R2A media without nitrogen sources but was negative on ARA test indicating no nitrogenase activity. The amplification of *nifH* gene was not possible and no *nif/fix* genes were found in the genome sequence of the bacteria. This suggests a limited ability for nitrogen fixation.

8.4.2. Presence of conserved PGPB genes in strain M24T3

In the genome of *Serratia* sp. M24T3 genes likely encoding for the ACC deaminase (WP_009638971); cellulase – β-glucosidase (WP_009635056; WP_009636821) and periplasmic β-glucosidase (WP_009638496); catalase (WP_009638086) and catalase/peroxidase (WP_009636268); pectinase - pectinesterase B (WP_009638872); superoxide dismutase (WP_009635546); chitinase (WP_009638226) and several genes involved in IAA, acetoin and 2,3-butanodiol biosynthesis were identified. No genes required for nitrogen fixation were found.

Three potential IAA biosynthesis pathways, the indole-3-acetamide (IAM), indole-3-acetonitrile (IAN) and indole-3-acetaldehyde pathways were identified in the genome of M24T3, and 10 genes might be involved in IAA biosynthesis. In the IAM pathway, tryptophan is converted to tryptamine by aromatic-L-amino-acid decarboxylase (WP_009635138), and indole-3-acetamide is converted to IAA by aliphatic amidase AmiE (WP_009634947, WP_009639043). In the IAN pathway, IAN is

converted directly to IAA by nitrilase (WP_009634944). Indole-3-acetoaldehyde is converted to IAA by aldehyde dehydrogenase (WP_009635369, WP_009635627, WP_009636235, WP_009637232, WP_009639021, WP_009639329).

For butanoate metabolism, eight genes were found in *Serratia* sp. M24T3 and might be involved in the production of acetoin and 2,3-butanediol. Pyruvate is converted to 2-(α-hydroxyethyl)-ThPP by pyruvate dehydrogenase (WP_009634667); ThPP (thiamine pyrophosphate) is converted to 2-(α-hydroxyethyl)-ThPP followed by conversion to 2-acetolactate by acetolactate synthase (WP_009635236; small subunit - WP_009634704 and large subunit - WP_009634705; small subunit - WP_009637883 and large subunit - WP_009637884). Moreover, acetoin is produced by two pathways, one from conversion of 2-acetolactate by alpha-acetolactate decarboxylase (WP_009635235), and another from conversion of diacetyl by acetoin reductase/2,3-butanediol dehydrogenase (WP_009635231). Acetoin is converted to 2,3-butanediol by acetoin reductase/2,3-butanediol dehydrogenase.

The phylogenetic tree of strain M24T3 closest ACC deaminase sequences indicated three main branches: sequences in one branch belong to the family *Enterobacteriaceae*, the other branch featured sequences from *Acidovorax* sp. KKS102 and *Acidovorax* sp. CF316 (class *Betaproteopacteria,* family *Comamonadaceae*), and the third branch showed mainly sequences from the genus *Pseudomonas* but included also one sequence from *Serratia rubidaea* AcdSPB1, *Klebsiella pneumoniae* AcdSPB2, *Bacillus cereus* AcdSPB4 and Uncultured bacterium clone mp16, respectively (Figure 8.1). ACC deaminase from *Serratia* sp. M24T3 showed more similarity to *Pantoea* sp. At-9b (WP_013512282) (94.9 %), *Pantoea* sp. AS-PWVM4 (WP_021183987) (94.2 %). The other two members from the genus *Serratia* with similarities in databases were *Serratia* sp. ATCC 39006 (WP_021013722) (91.2 %) and *Serratia rubidaea* AcdSPB1 (AEQ29824) (86.3 %) (Figure 8.1). These results suggest that the ACC deaminase gene is not conserved only in members of genus *Serratia*.

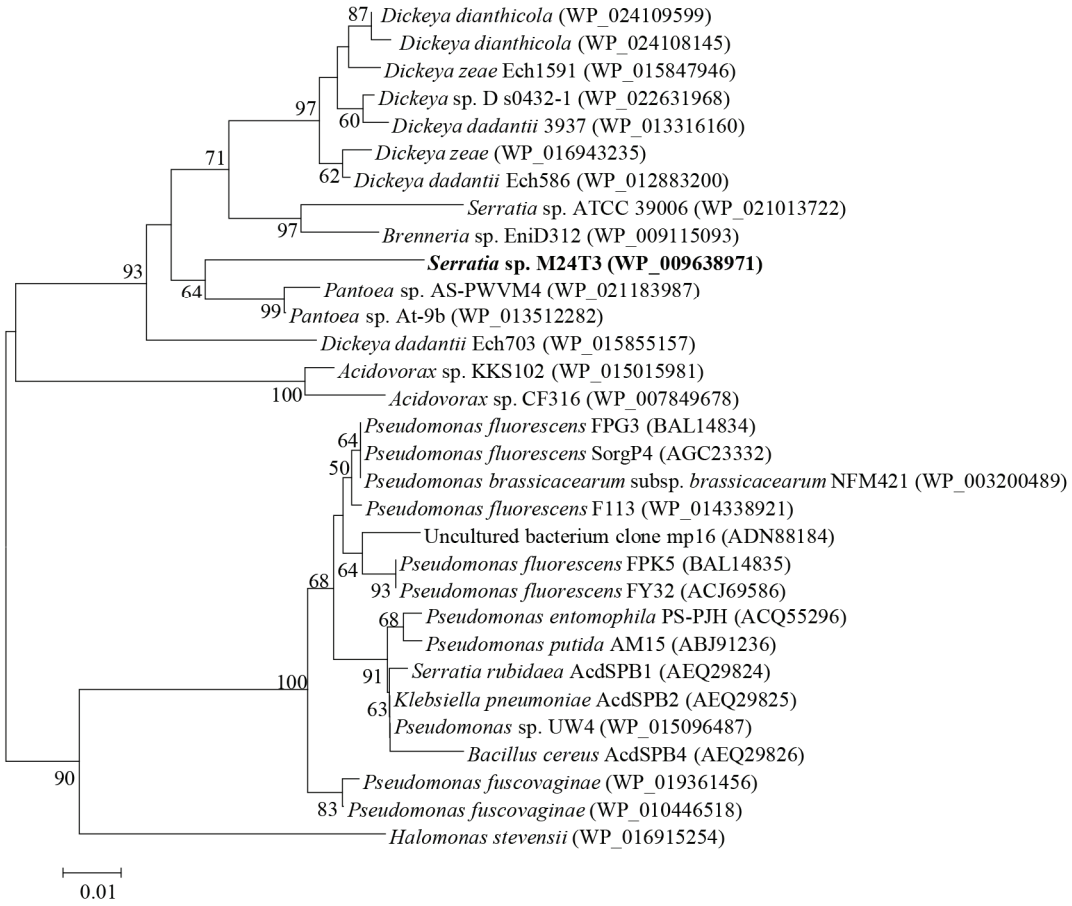

Figure 8.1. Dendrogram of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The tree topology was obtained by Maximum Likelihood analysis of protein sequences in MEGA 5, previously aligned by CLUSTALW. Reference protein sequences were obtained from NCBI database (cutoff of 86 %) and sequence from *Serratia* sp. M24T3 is indicated in bold. The numbers indicate the percentages of bootstrap sampling, derived from 1,000 replications, values below 50% are not shown. Scale bar represents 1% sequence divergence.

8.4.3. Multiple effects of strain M24T3 on plant development

Seed germination was inhibited when the strain M24T3 was inoculated into *A. thaliana* seeds when compared with uninoculated controls (results not shown). Moreover, bacterial suspension and bacterial supernatant of strain M24T3, transformed with plasmid pHRGFPGUS and wild type, promoted an apparent decrease on the primary root elongation (Figure 8.2). However, the number of lateral roots seemed to be increased (Figure 8.3) and that is an effect that might be related to the production of indolic compounds (IAA) produced by strain M24T3.

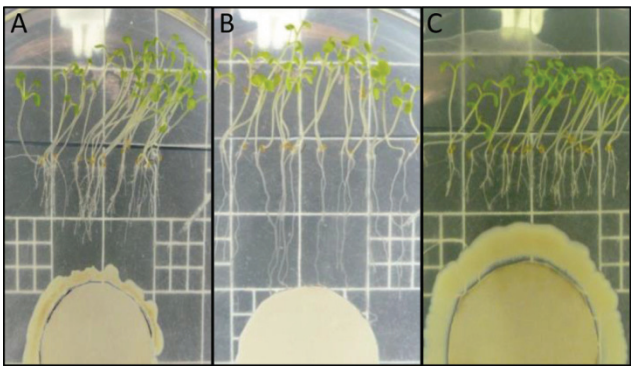

Figure 8.2. Alteration of root elongation in *Arabidopsis thaliana* seedlings by *Serratia* sp. M24T3. Seeds were germinated in a line on half concentrated MS agar and 50 µl of bacterial suspensions were added on sterile paper. Primary root elongation was inhibited by strain M24T3 wild type (A) and transformed (C) suspensions. The root elongation in control with LB broth was not inhibited (B).

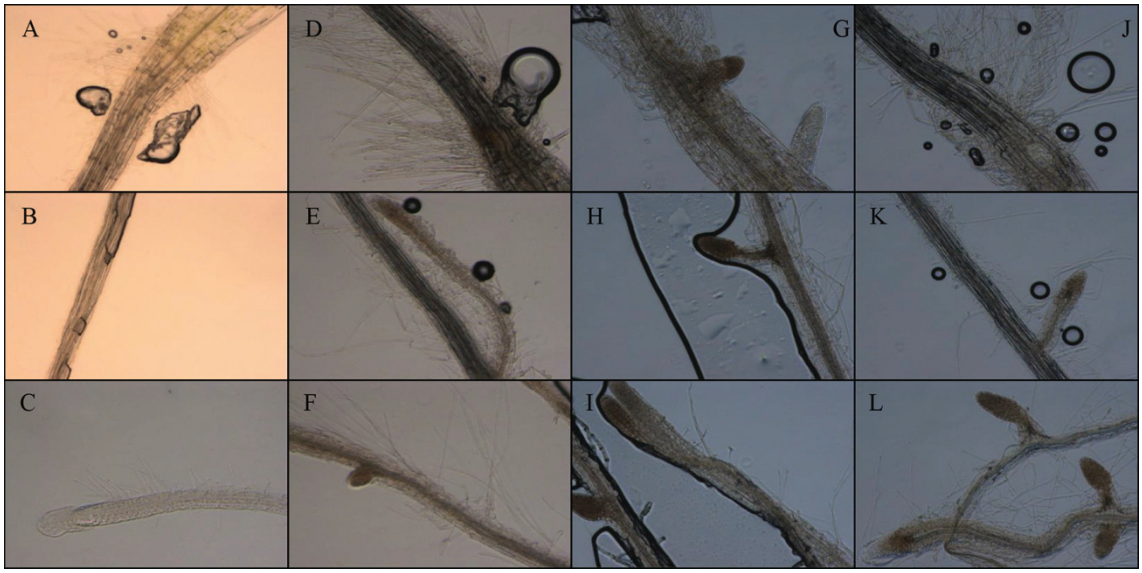

Figure 8.3. Light microscopy observation of *A. thaliana* roots (100 x). Roots removed from: control with LB broth (A-C); suspension of strain M24T3 transformed (D-F); supernatant of strain M24T3 transformed (G-I); suspension of strain M24T3 wild type (J-L).

8.4.4. Endophytic colonization of *Serratia* sp. M24T3 in *Arabidopsis*

After bacterial inoculations, plantlets of *A. thaliana* were grown in liquid MS medium. The colonization of strain M24T3 was microscopically observed. There was visible evidence for bacterial colonization (data not shown). Using CLSM it was also possible to confirm effective colonization of *gfp*/*gusA*-tagged *Serratia* sp. M24T3 in *A. thaliana* intercellular spaces (Figure 8.4A) in the cortex region as well as vascular zones (Figure 8.4B). The localization of the M24T3 (*gfp*/*gusA)* cells was also supported by the fact that the chlorophylls were located in the same optical section. The *gfp*/*gusA*-tagged *E. coli* negative control showed no endophytic colonization (Figure 8.4C). Thus, *Serratia* sp. M24T3 behaved as a competent endophyte.

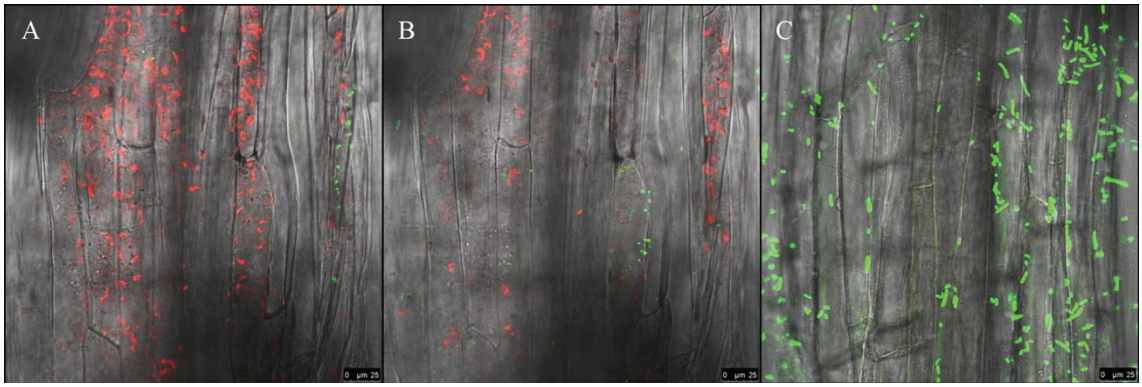

Figure 8.4. Confocal laser scanning microscopy micrographs of *gfp*/*gusA*-tagged *Serratia* sp. M24T3 cells colonizing *A. thaliana* roots (A and B). A *gfp*/*gusA*-tagged *E. coli* strain was used as colonization negative control (C). Longitudinal section of *A. thaliana* root inoculated with strain M24T3 and grown for 7 days in a 12 multiwell plate. Fluorescence was observed after colonizing intercellular spaces (A) in the cortex region as well as vascular zones (B) (Bar 25 µm).

8.5. Discussion

As the climate changes, new biological strategies for sustainability and pest control are among the concerns of today for a near future. Bacteria in their natural habitats colonize the plants inside and outside (endophytes and epiphytes) and proved to have a positive, neutral or negative role to the plants. Therefore, they have been used as potential new tools in sustainable agriculture. Several studies reported mechanisms of plant growth promotion by PGPB and their beneficial role for plants (Gamalero & Glick, 2011; Glick *et al.*, 2007; Hardoim *et al.*, 2008; Reinhold-Hurek & Hurek, 2011; Ryan *et al.*, 2008; Stoltzfus *et al.*, 1997). Moreover, some bacteria establish a close relation with their host, for example *Gluconacetobacter diazotrophicus* Pal5 with sugarcane and rice plants (Meneses *et al.*, 2011; Rouws *et al.*, 2010). Similarly, the colonization capability of nematicidal *Serratia* sp. M24T3 in *A. thaliana* evaluated here was characterized for its ability to produce substances involved in plant growth promotion and its genome, sequenced in a previous project, was queried for genes recognized as to be involved in plant growth promotion.

Serratia sp. M24T3, according to sequence analysis of 16S rRNA gene, is a close relative of *S. plymuthica*, considered non-pathogenic (Chapter 7; Proença *et al.*, 2012a). Several studies on *S. plymuthica* have shown its PGPB role as well as its ability to act against pathogenic *Agrobacterium* (Dandurishvili *et al.*, 2011; Neupane *et al.*, 2012c). Strains from the species *S. plymuthica* are ubiquitous and have been preferentially recovered from rhizospheres, both as free-living and endophytic organisms. This species acts by antibiosis limiting the presence of other microorganisms inside the plant, by production of lytic enzymes, by induction of plant defense mechanisms, and by competition for nutrients with other colonizers, such as iron through secretion of siderophores (de Vleesschauwer & Höfte, 2007). As the closest type strain of *S. plymutica*, *Serratia* sp. M24T3 produces siderophores, lipases but no proteases (Chapter 4 and 6; Paiva *et al.*, 2013; Proença *et al.*, 2010).

Strain M24T3 solubilized phosphate and zinc, and these findings demonstrated that the strain might support plant growth, since P and Zn are essential macro and micronutrients, respectively, for plant growth (Gamalero & Glick, 2011). On the other hand, zinc solubilization by *G. diazotrophicus* PAL5 promoted the nematicidal activity against root knot nematode *Meloidogyne incognita* (Saravanan *et al.*, 2007). Therefore, *Serratia* sp. M24T3 ability to solubilize Zn may be part of the mechanism of toxicity

against the PWN demonstrated by the strain *in vitro* (Chapter 6, Paiva *et al.*, 2013). Other strategies may be involved in the toxicity against the nematode in this strain since the draft genome sequence showed multiple genes potentially involved in virulence and nematotoxicity such as the genes coding for colicin V and bacteriocin biosynthesis (Chapter 7; Proença *et al.*, 2012a).

Microbial cellulases are involved in plant cell wall degradation. These enzymes facilitate invasion of microbial cells into plant cells and, depending on the amount produced by the bacteria can potentiate the endophytic colonization or, render bacteria plant pathogenic (Reinhold-Hurek *et al.*, 2006). Here, *Serratia* sp. M24T3 showed cellulolytic activity, the ability for CMC degradation, which was also supported by genomic information with genes encoding for cellulase – β-glucosidase (WP_009635056; WP_009636821) and periplasmic β-glucosidase (WP_009638496) and pectinase - pectinesterase B (WP_009638872) present. The cellulolytic activity was low and the phosphate or zinc solubilization activity was high, supporting the idea of strain M24T3 being a PGPB, but not a phytopathogen (Reinhold-Hurek *et al.*, 2006). Besides, cellulases might be also involved in the pathogenesis process of PWN to pine trees (Kikuchi *et al.*, 2011).

Several endophytic bacteria are involved in biological nitrogen fixation (BNF) (Reinhold-Hurek & Hurek, 2011). *Serratia* sp. M24T3 did not possess nitrogenase activity and it was not possible to detect a *nifH* gene in the strain's genome sequence. On the other hand, strain M24T3 showed a gene coding for nitrilase, an enzyme involved in hydrolysis of nitrile compounds (such as IAN) resulting in production of ammonia as well as involved in several plant-microbe interactions (Howden & Preston, 2009).

For studying plant-microbe interactions, several studies used reporter genes, for example the *gfp* gene for green fluorescent protein (Ramos *et al.*, 2002; Rouws *et al.*, 2010). In our study, *Serratia* sp. M24T3 carrying the pHRGFPGUS plasmid showed green fluorescence inside *A. thaliana* intercellular spaces, in the cortex region as well as vascular zones. These results suggest efficient plant colonization obtained with *gfp*/*gusA*-tagged *Serratia* sp. M24T3. Moreover, germination of *A. thaliana* seeds was inhibited in contact with bacterial growth. On the other hand, suspension and supernatant of strain M24T3 with plasmid pHRGFPGUS and wild type, inhibited the primary root elongation but increased the number of lateral roots. In a previous work,

Serratia marcescens strain 90-166, the plant growth-promoting rhizobacterium (PGPR), showed inhibition of primary roots and enhancement of lateral roots in *A. thaliana*, an effect caused by IAA produced by the bacteria (Shi *et al.*, 2010). Such alterations might be beneficial for plants for increased water absorption and nutrients uptake (Patten & Glick, 2002). Therefore, the effects induced in the plant by strain M24T3 (inhibition of the primary root elongation but increase in the number of lateral roots) might be due to production of IAA. The ability of the strain to produce IAA was confirmed by biochemical tests *in vitro* and is supported by the presence of 10 genes in the genome that might be involved in IAA biosynthesis pathways. When querying the KEGG database, genes encoding for aldehyde dehydrogenase were present in all genomes available for *Serratia* spp. Aliphatic amidase *amiE* genes were present just in *S. proteamaculans* and *S. liquefaciens* but nitrilase genes were not previously found in any *Serratia* genome sequences. Thus, this nitrilase (WP_009634944) from *Serratia* sp. M24T3 constitutes the first report of this enzyme in the genus *Serratia*. However, the concentration of IAA produced by *Serratia* sp. M24T3 was basically the same when grown in LB broth supplemented with and without L-tryptophan. This suggests and supports the genomic information that the IAA synthesis pathway is independent of L-tryptophan.

Ethylene is a stress-induced plant hormone involved in inhibition of plant growth but at low levels is also required for normal plant development (Morgan & Drew, 1997). The gene encoding for ACC deaminase is present in *Serratia* sp. M24T3 and might be involved in lowering ethylene levels by cleaving the ethylene precursor ACC into ammonia and α-ketobutyric acid (Glick *et al.*, 1998). The ACC deaminase present in *Serratia* sp. M24T3 showed high similarities with ACC deaminases from *Pantoea* spp. but the sequence was just found in two other *Serratia* strains based in NCBI/KEEG databases.

Strain M24T3 has genes encoding for catalase and SOD that can be part of the mechanism of bacterial protection against ROS release from plants, and which can promote an effective bacterial colonization of the plant. Moreover, the genome sequence of *Serratia* sp. M24T3 contains a gene encoding for chitinase and previous work showed its antifungal importance (Vaidya *et al.*, 2001). These genetic informations are supported by positive results from the biochemical assays.

Previously, the bacterial volatiles, acetoin and 2,3-butanediol, produced by PGPR showed to have an important role in plant-growth promotion of *Arabidopsis* (Ryu *et al.*, 2003) and could be involved in plant protection against fungal and bacterial infections (Chapter 7; Proença *et al.*, 2012a). The genome of *Serratia* sp. M24T3 harbors eight genes that might be related to butanoate metabolism and involved in production of acetoin and 2,3-butanediol. However, the acetoin reductase/2,3-butanediol dehydrogenase was just found in half of the genome sequences of *Serratia* strains included in KEGG database. Furthermore, this enzyme was found only in genome sequences of strains close to *S. plymuthica*.

In conclusion, strain M24T3 showed potential as PGPB in experiments with the plant model *Arabidopsis*, supported by biochemical characteristics determined with tests *in vitro* and also supported on genomic analysis. Furthermore, the results with the plant model showed that *Serratia* sp. M24T3 was a competent endophyte. The plasmid pHRGFPGUS was an important tool to label strain M24T3 for monitoring the bacterial colonization of *A. thaliana*, without causing harm to the plant. Thus, *Serratia* sp. M24T3 is able to colonize another plant species beyond pine trees. These data together with previous results concerning nematicidal activity, makes the strain M24T3 very promising as an alternative for agrochemicals as pesticides and for fertilizers thus reducing the economic and environmental foot-print of agriculture.

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Concluding Remarks

The major conclusions from the results presented in this thesis and their significance in understanding of PWD as a complex plant disease are summarized below.

In **Chapter 2**, Bacteria and Archaea were found, for the first time, living together as part of the same endophytic community of *P. pinaster* trees. The endophytic bacterial community from trees from the areas of Malhada and Avô, detected by cultivable methods, comprised eight bacterial classes, namely *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria*, *Flavobacteriia*, *Gammaproteobacteria* and *Sphingobacteriia*. In both sampling areas, the most abundant bacterial class was the *Gammaproteobateria*. The family *Acidobacteria* was only found in trees from Avô and strains from the family *Flavobacteriia* were only isolated in Malhada's trees. Conversely, when the diversity was evaluated by DGGE clones, nine classes were found in Avô the area and 12 classes were found in the Malhada area. Comparatively, two Classes (*Flavobacteriia* and *Verrumicrobiae*) were only found in Avô and five Classes (*Bacteroidia*, *Deinococci*, *Fusobacteria*, *Planctomycetaceae* and *Spirochaetes*) were only found in Malhada. Comparison between bacterial isolates and DGGE band clones showed that, additionally, six classes were found by molecular approaches - *Bacteroidia*, *Deinococci*, *Fusobacteria, Planctomycetaceae*, *Spirochaetes* and *Verrumicrobiae*. However, cultivation methods made it possible to isolate and identify some bacterial species not detected by DGGE, probably comprising minority groups that escaped detection in global assessments because of limitations of PCR-DGGE. These species belonged to the families *Mycobacteriaceae*, *Patulibactereaceae*, *Alcaligenaceae*, *Nevskiaceae and Cytophagaceae* from the Malhada area; to the families *Dermacoccaceae* and *Phyllobacteriaceae* from the Avô area; and *Caulobacteraceae* and *Chithinophagaceae* from both sampling areas. None of the ten most important plant pathogenic species, according to scientific/economic importance, were isolated or detected by 16S rRNA gene sequence in this study. On the other hand, species detected in both sampling areas were described to be associated with plants; roles in plant growth promotion have been suggested. Archaea were only identified by DGGE profiling methods but not by cultivation. All DGGE band clones from both sampling areas revealed novel sequences (i.e. < 97 % identity with cultivated archaea) suggesting that the endophytic archaeal community of pine trees is

quitenovel. DGGE band clones have close similarity with uncultured archaeal clones that were previous reported as part of archaeal community from water samples and include members of the phylum *Euryarchaeota* – archaea of the South African Goldmine Group and Anaerobic Methanotrophic archaea; the phylum *Thaumarchaeota* – Marine Group I and to the phylum *Crenarchaeota* – miscellaneous crenarchaeote group. DGGE fingerprinting of bacteria or archaea did not reveal a specific endophytic microbial community profile for PWD. Moreover, the presence of *nifH*, *nifJ*, *nirS* and *nirK* genes in the endophytic bacterial community supports the possibility of N_2 fixation inside of trees, and also denitrification, with the production of the metabolic relevant intermediary NO. The diversity of *nifH* and *nifJ* genes suggested that the population of N_2 fixing bacteria was diverse within the *Gamma-* and the *Alphaproteobacteria*, which were also the main groups detected by 16S rRNA gene sequence, with some of the clones only detected in one of the sampling areas. The prevalence of *nir* genes seemed to be more related with the sampling area and the structural diversity of the endophytic community (only *nirK* in Avô, mostly *nirS* in Mallhada) and not with the physiological status of the trees. Statistical analysis by redundancy analysis (RDA) revealed that the endophytic community of the pine trees was dependent on the sampling areas and the presence of pinewood nematode. *B. xylophilus* apparently showed a close relationship with families *Enterobacteriaceae* and *Xanthomonadaceae*. The diversity of the culturable endophytic community in both areas was higher, for pine trees with symptomatic stage equal or higher than PWD symptom class sII where the nematode was detected, than for healthy trees, with symptomatic stage equal or lower than SII and without nematodes. In addition, Shannon-Weaver indices as indicator for bacterial and archaeal species diversity were higher for symptomatic trees than for healthy trees when calculated for DGGE profiles from Avô. Noteworthingly, DGGE profiles from pine trees from Malhada showed higher diversity in healthy than in symptomatic trees and archaea were only detected in symptomatic trees from Malhada area.

In **Chapter 3**, strain A37T2^T, isolated from endophytic microbial community of *P. pinaster* in **Chapter 2**, was characterized and described as new species *Chitinophaga costaii* A37T2^T. This species belongs to the genus *Chitinophaga* (family *Chitinophagaceae*, phylum *Bacteroidetes*).

In **Chapter 4**, the diversity of bacteria associated with PWN and other nematodes (Families *Rhabditidae* and *Aphelenchoididae*) from *Pinus pinaster* trees with PWD was for the first time identified in Portugal. The number of isolated bacteria and the species isolated were not related with the symptomatology or with the diameter of sampled trees measured at breast height. The bacteria associated with PWN in Portugal were mainly *Pseudomonas*, *Burkholderia*, and *Enterobacteriaceae* (genera *Yersinia*, *Serratia*, *Ewingella*, *Pantoea* and *Erwinia*). The genus *Burkholderia* associated with PWN was for the first time reported. Only one Gram-positive strain (*Actinobacteria*) was found. No single isolated bacterial species associated with *B. xylophilus* and other nematodes was common to all of the different forest areas sampled, but only strains from the genus *Pseudomonas* were found in all sampling sites. Biochemical characterization showed that bacteria were able to produce siderophores (60-100%), proteases activity (0-30%), and lipases activity (10-100%), according to sampled areas. Therefore, the abilities to produce siderophores and lipases by most isolates may contribute for these bacteria to have a role in plant physiological response.

Bacteria associated with PWN in **Chapter 4** were found as part of endophytic bacterial community in **Chapter 2**. This result suggests that most probably the bacteria associated with PWN are endophytes of *P. pinaster* trees.

In **Chapter 5**, *B. xylophilus* was detected in nine symptomatic pine trees from three-fifths of sampling areas in Nebraska, USA. The bacterial strains carried by PWN belonged mainly to the class *Gammaproteobacteria* (79.9 %). The classes *Betaproteobacteria* (11.7 %), *Bacilli* (5.0 %), *Alphaproteobacteria* (1.7 %) and *Flavobacteriia* (1.7 %) were identified. Strains from the genera *Chryseobacterium* (class *Flavobacteriia*) and *Pigmentiphaga* (class *Betaproteobacteria*) were found associated with the nematode for the first time. The isolates from the United States, Portugal and China belonged to 25 different genera and only strains from the genus *Pseudomonas* were found carried by PWN from all countries. Nevertheless, the strains from China were closely related to *P. fluorescens* and the strains isolated from Portugal and USA were phylogenetically related to *P. mohnii* and *P. lutea*.

Both **Chapters 4** and **5** showed that the major bacterial population associated to the nematodes differed according to the forest area where the nematodes were

sampled and none of the bacterial species was found in all different forest areas.

In **Chapter 6**, twenty-one of the forty-seven strains tested showed a capacity to produce extracellular products with 100 % of nematicidal activity. Five belonged to the species *S. plymuthica*, five to *P. lutea*, three to *S. marcescens*, two to *R. aquatilis* and one strain to each of the species *P. koreensis*, *P. constantinii*, *P. putida*, *P. moorei*, *P. cypripedii* and *C. pusillum*. All *Burkholderia* strains were non-toxic. In contrast, all *Serratia* strains except one, exhibited high toxicity. Nematodes incubated with *Serratia* strains showed, by scanning electron microscope (SEM) observation, deposits of bacteria on the nematode cuticle. *Serratia* sp. A88copa13, the most effective nematicidal strain as well as with the highest activity in the zymogram gel, showed an extracellular serine protease with 70 kDa as the major responsible for the toxicity of the supernatant. This extracellular serine protease is phylogenetically different, in size and biochemically from previously described proteases. The protease had diverse nematicidal activities on different species of *Bursaphelenchus* which make it useful as primary screening against other nematodes.

In **Chapter 7**, the genome of the two bacterial strains with nematicidal activity assessed in **Chapter 6**, *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1 were sequenced. Both, *Serratia* sp. M24T3 and *Pseudomonas* sp. M47T1 genome sequences carry multiple genes potentially involved in nematotoxic activity such as Colicin V and Bacteriocin biosynthesis and a gene that contains a bacterial RTX (repeats in the structural toxins) toxin-activating protein-C signature. A gene encoding for nitrogen regulatory protein P-II was also present in both genome sequences and might be important for plant growth-promotion. In the *Serratia* sp. M24T3 genome sequence, the *yafQ* gene encoding for an endoribonuclease (YafQ toxin) is potentially involved in nematicidal activity. Moreover, the *YdhE*/*NorM* gene encoding for a homolog of a multidrug and toxin extrusion (MATE) family efflux pump and three genes coding for Rhs family protein that may be involved in adhesion or virulence were found in the genome sequence of *Pseudomonas* sp. M47T1. Plant-growth promoting bacterial genes were also found in both genome sequences.

In **Chapter 8**, biochemical characterization of *Serratia* sp. M24T3 showed positive results for phosphate and zinc solubilization, cellulolytic and chitinolytic activity,

production of siderophores, no proteolytic activity, catalase and superoxide dismutase (SOD), production of indole acetic acid (IAA) and no nitrogenase activity (neither *nif*/*fix* genes). In genome of *Serratia* sp. M24T3 genes encoding for the 1-aminocyclopropane-1-carboxylate (ACC) deaminase; cellulase – β-glucosidase and periplasmic β-glucosidase; catalase and catalase/peroxidase; pectinase - pectinesterase B; SOD and chitinase. Moreover, ten genes involved in IAA, acetoin and 2,3-butanodiol biosynthesis were found. Strain M24T3 showed a gene coding for nitrilase, an enzyme involved in hydrolysis of nitriles compounds (such as indole-3-acetonitrile) that results in production of ammonia as well as is involved in several plant-microbe interactions. In addition, this constitutes the first report of this enzyme in the genus *Serratia*. The presence of *gfp*-tagged M24T3 inside root tissue, intercellular spaces, inside plant cells and vascular zones was confirmed by confocal laser scanning microscopy. The strain promoted the decrease in plant root elongation but increased the number of lateral roots, an effect probably related with the production of IAA that might be beneficial for plant. The production of IAA by *Serratia* sp. M24T3 was independent of L-tryptophan as suggested by the genomic information on the IAA synthesis pathway. The ACC deaminase gene was found in the M24T3 genome, supporting that the strain can promote plant growth by lowering plant ethylene. *Serratia* sp. M24T3 seems to be useful as a PGPB according to the results obtained with the plant model, supported by results of the biochemical characterization *in vitro* and on genomic analysis.

Overall, the results presented in this thesis provided a broad and comprehensive assessment of the microbial diversity and functionality associated with Pine Wilt Disease. Bacteria and archaea were found to be part of the endophytic microbial community of *P. pinaster* trees and might have a role inside the trees. Moreover, the diversity of bacteria carried by PWN was characterized and some of these bacteria were demonstrated to have nematicidal activity against PWN. Two strains of genus *Serratia* with nematicidal activity were studied in more detail as part of this thesis: *Serratia* sp. A88copa13 showed a serine protease with 70kDa possibly a major factor for nematotoxic activity in this strain and *Serratia* sp. M24T3, when analyzed for its plant-bacteria interaction, showed to be a potential PGPB.

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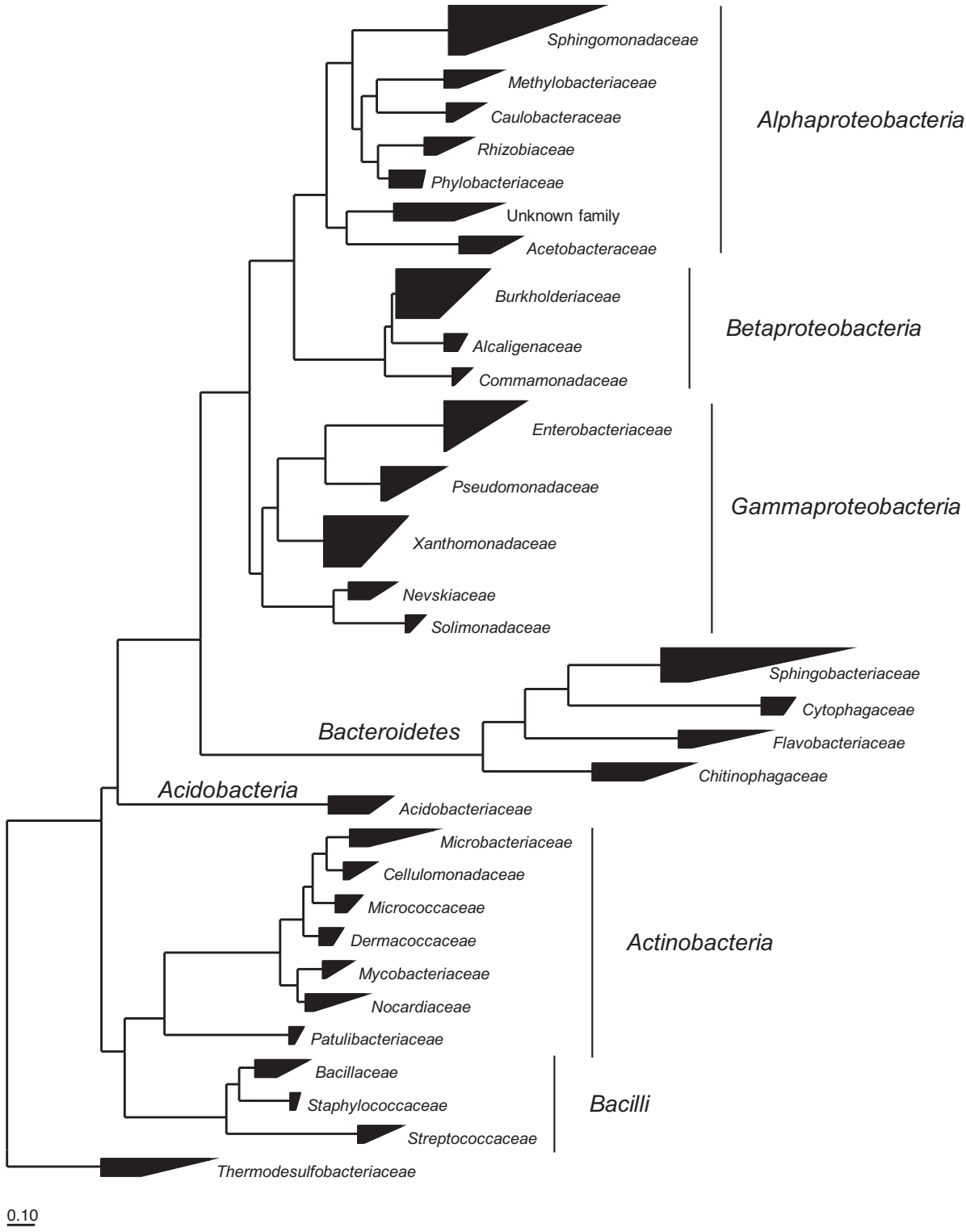
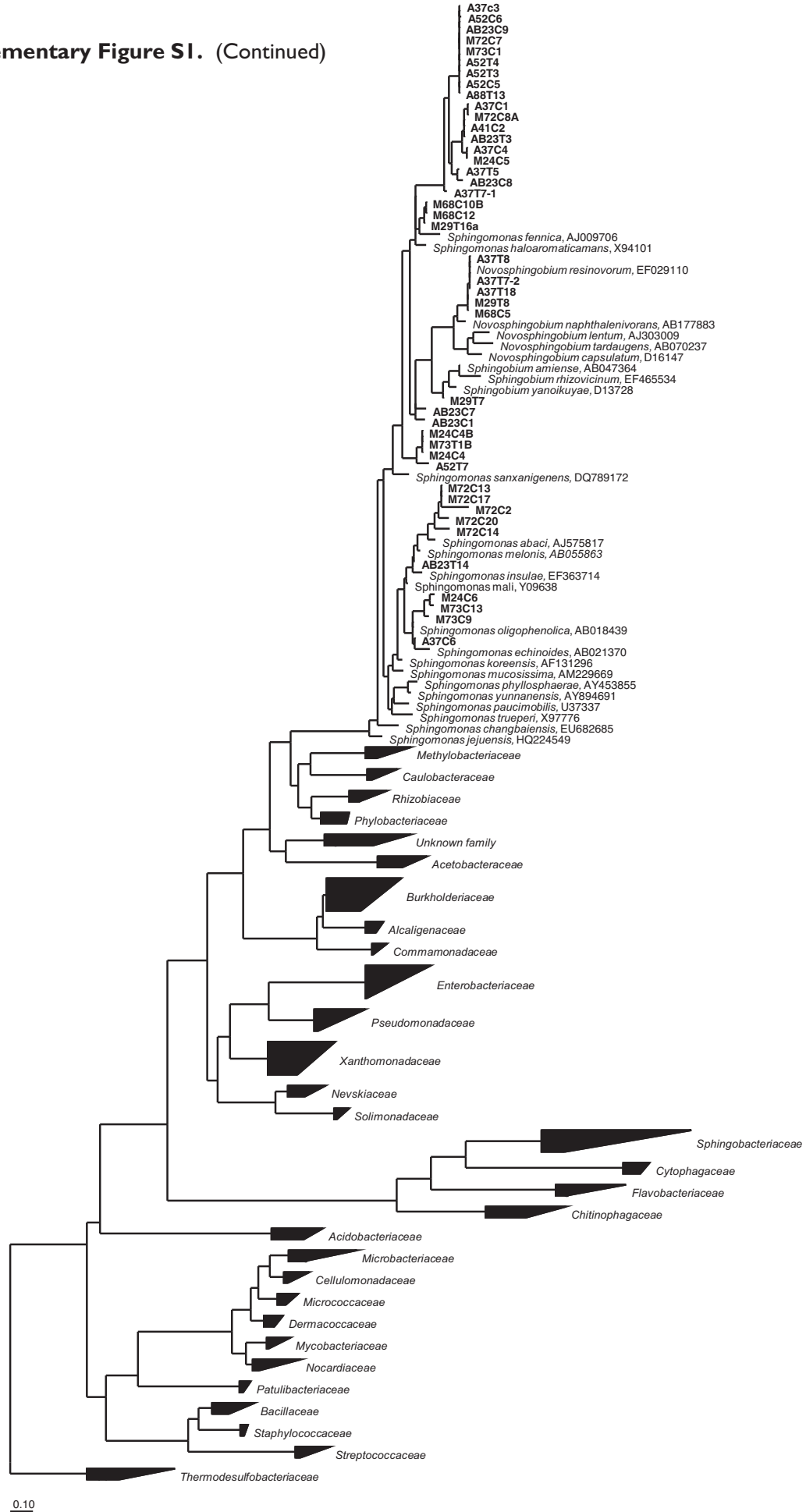
Appendix

A



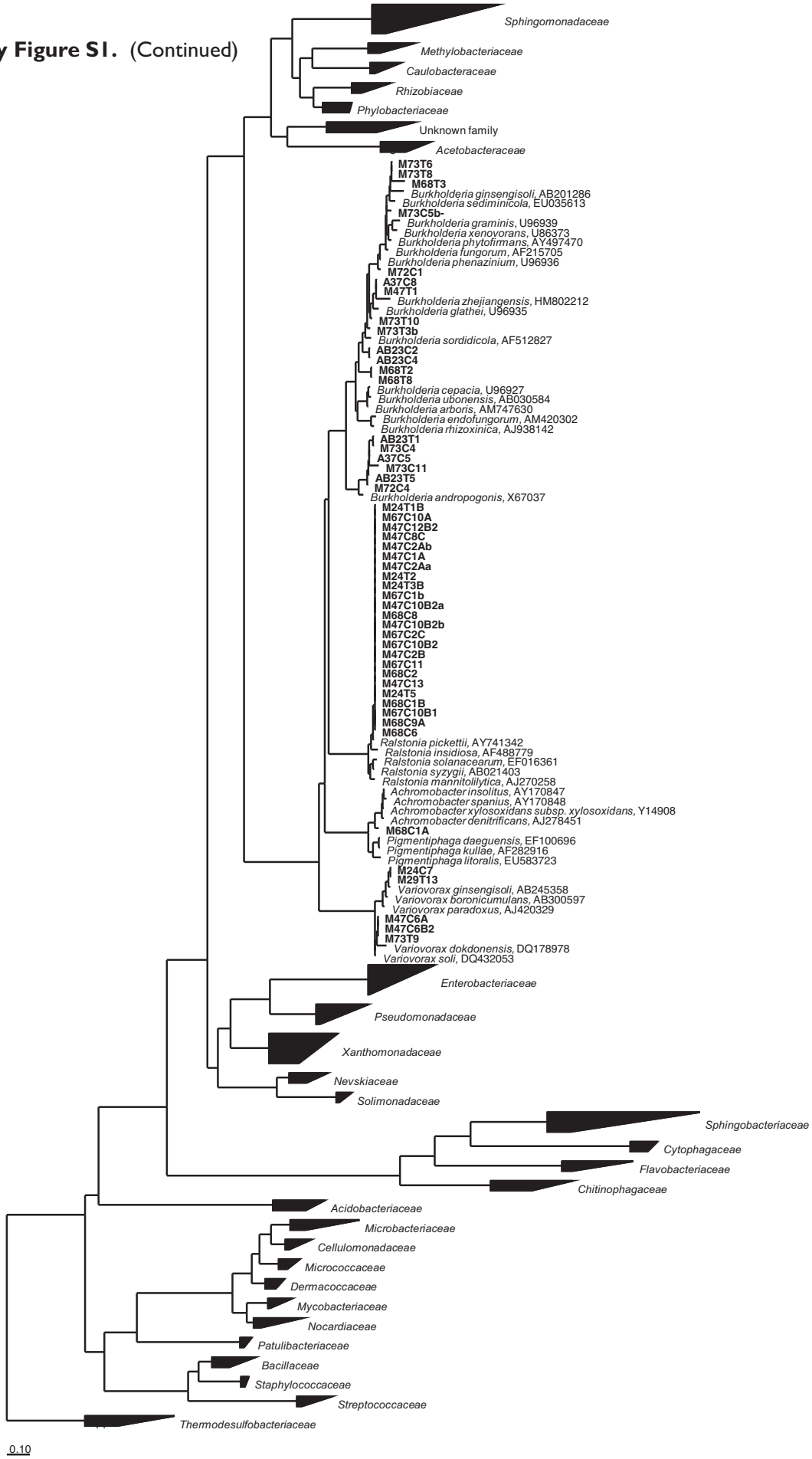
Supplementary Figure S1. Phylogenetic analysis of bacterial 16S rRNA gene sequences of endophytic bacterial isolates from pine trees. The dendrogram was constructed by the RAxML method with GTRGAMMA model included inside ARB software. The phylogenetic tree represents the classes *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacilli* and the phylum *Bacteroidetes*, discriminates the families identified in this study and is rooted by the family *Thermodesulfobacteriaceae*. The following phylogenetic trees in next pages are the extension of this families clusters. The isolates in this study are indicated in bold, comprise the isolates from areas Avô and Malhada (starting with A or M, respectively) and were compared with type strain of each species. Scale bar, 10 inferred nucleotide substitutions per 100 nucleotides.

Supplementary Figure S1. (Continued)



Supplementary Figure S1. (Continued)

Supplementary Figure S1. (Continued)



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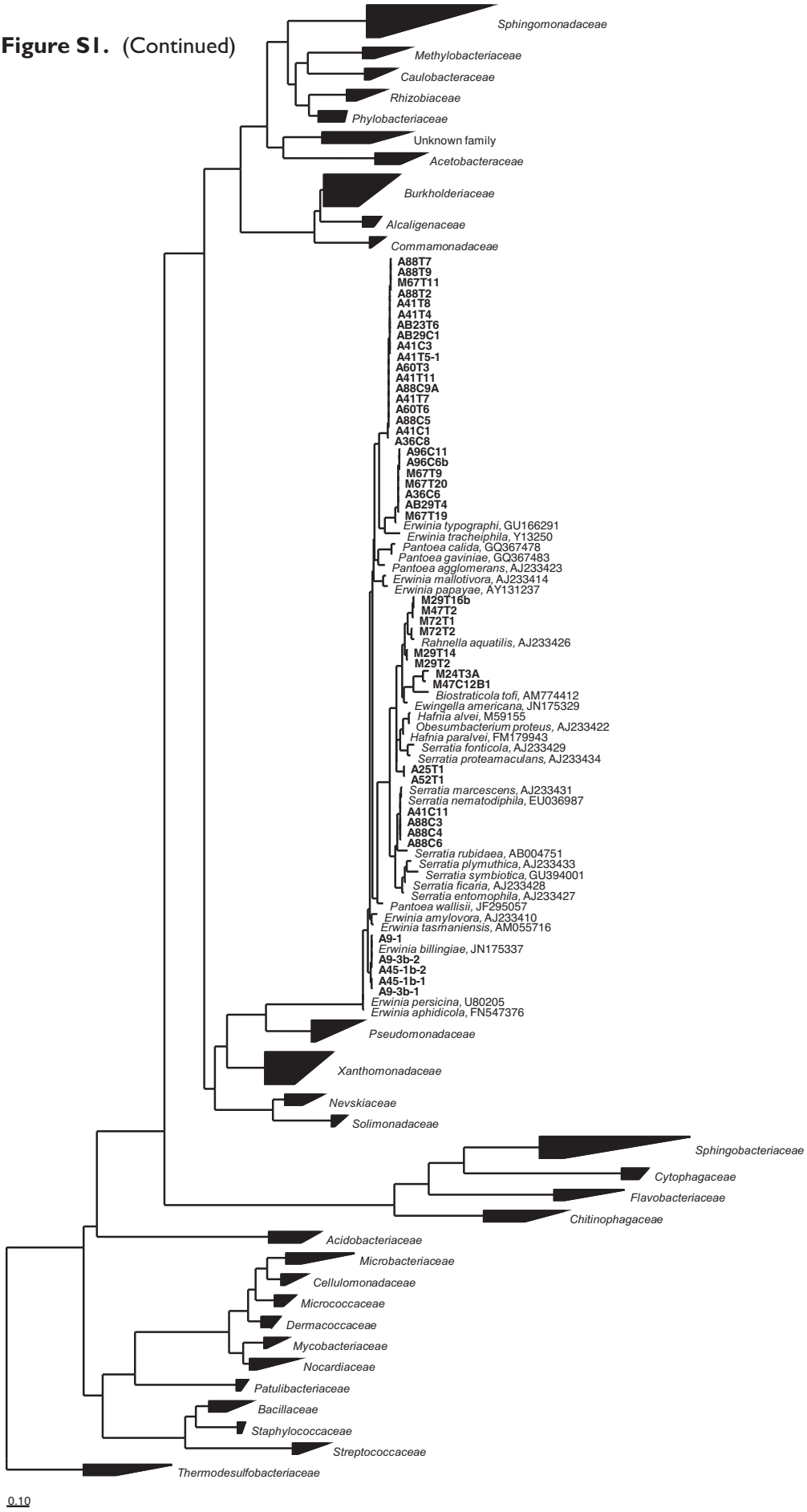

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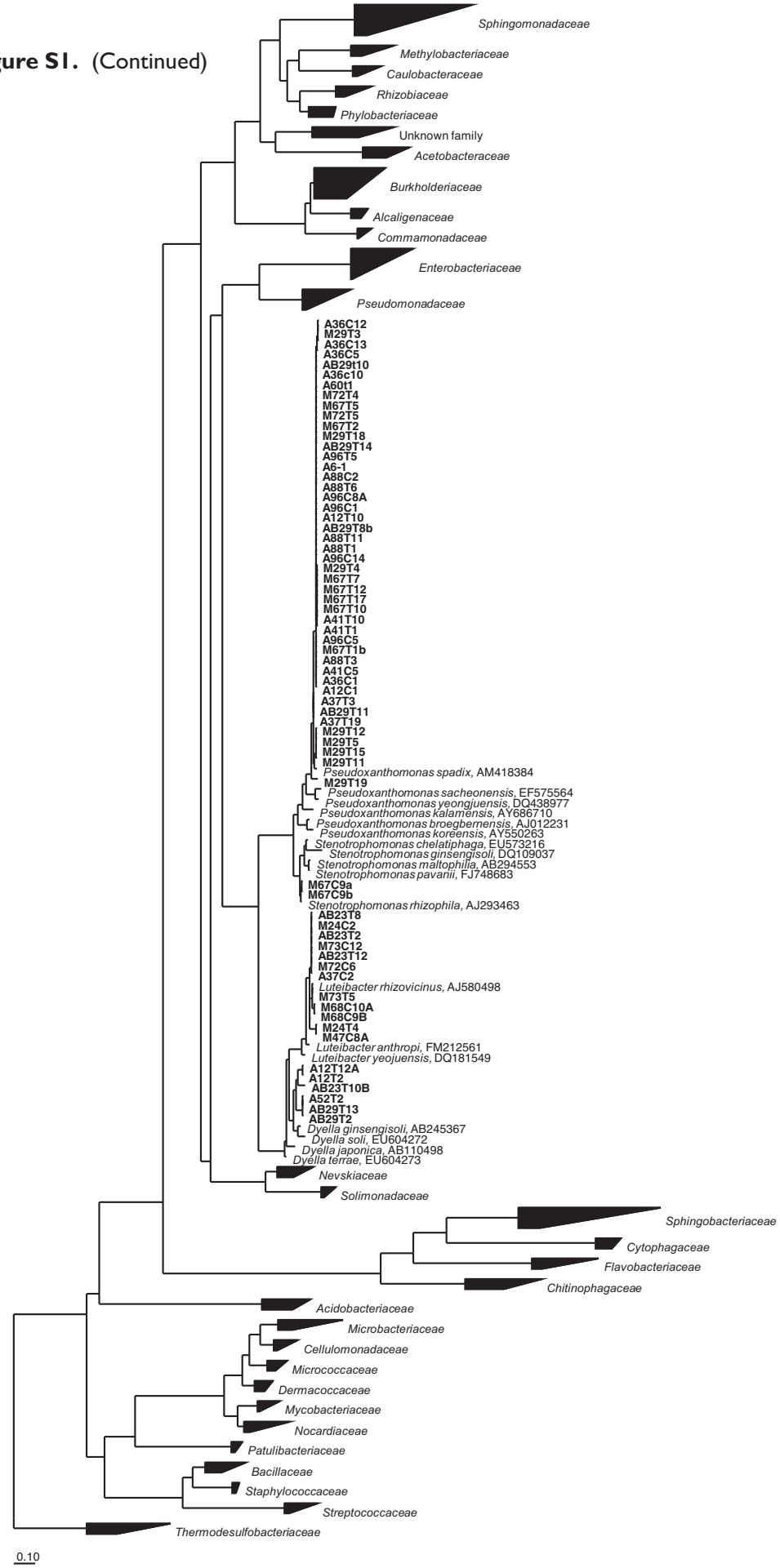

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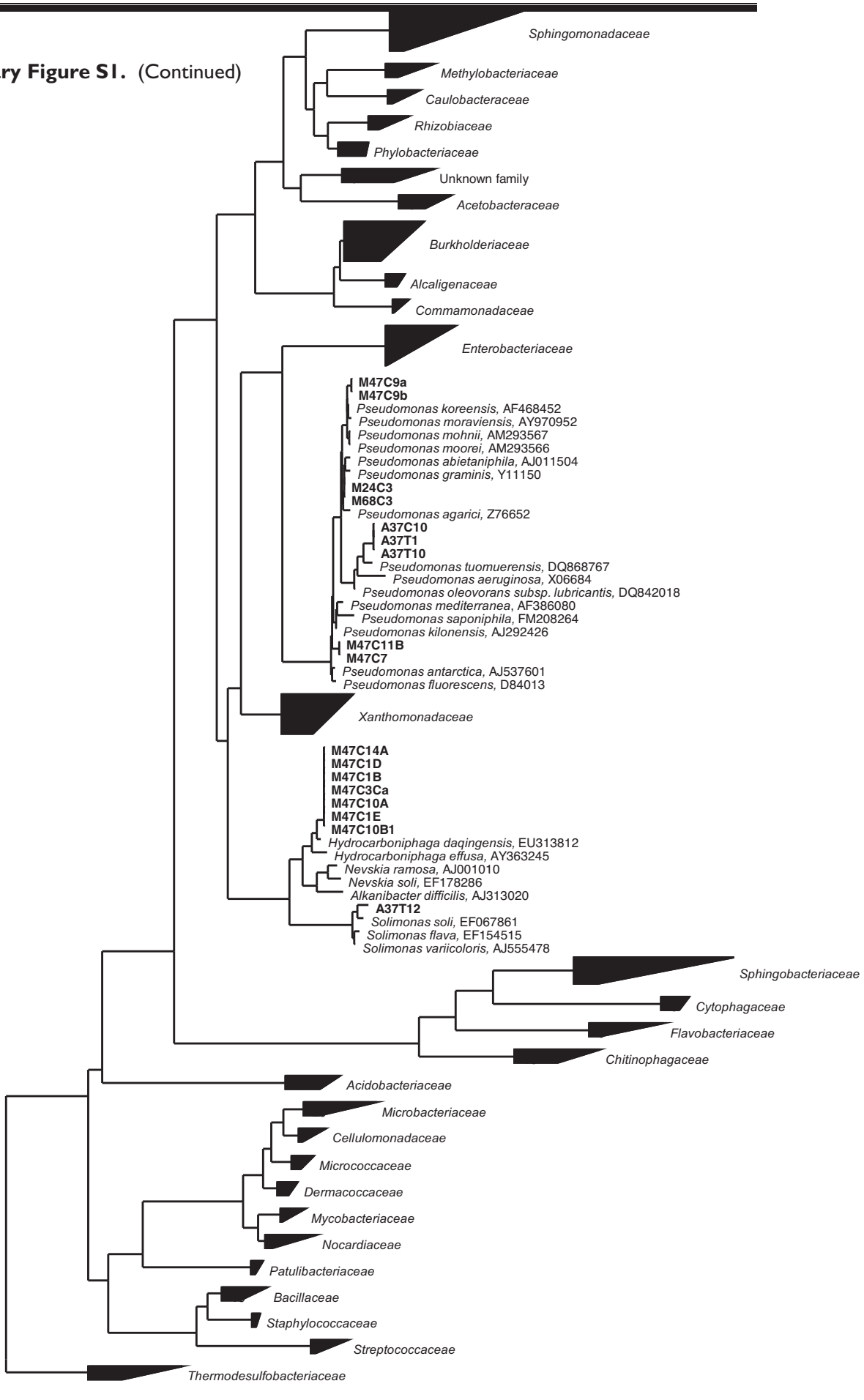

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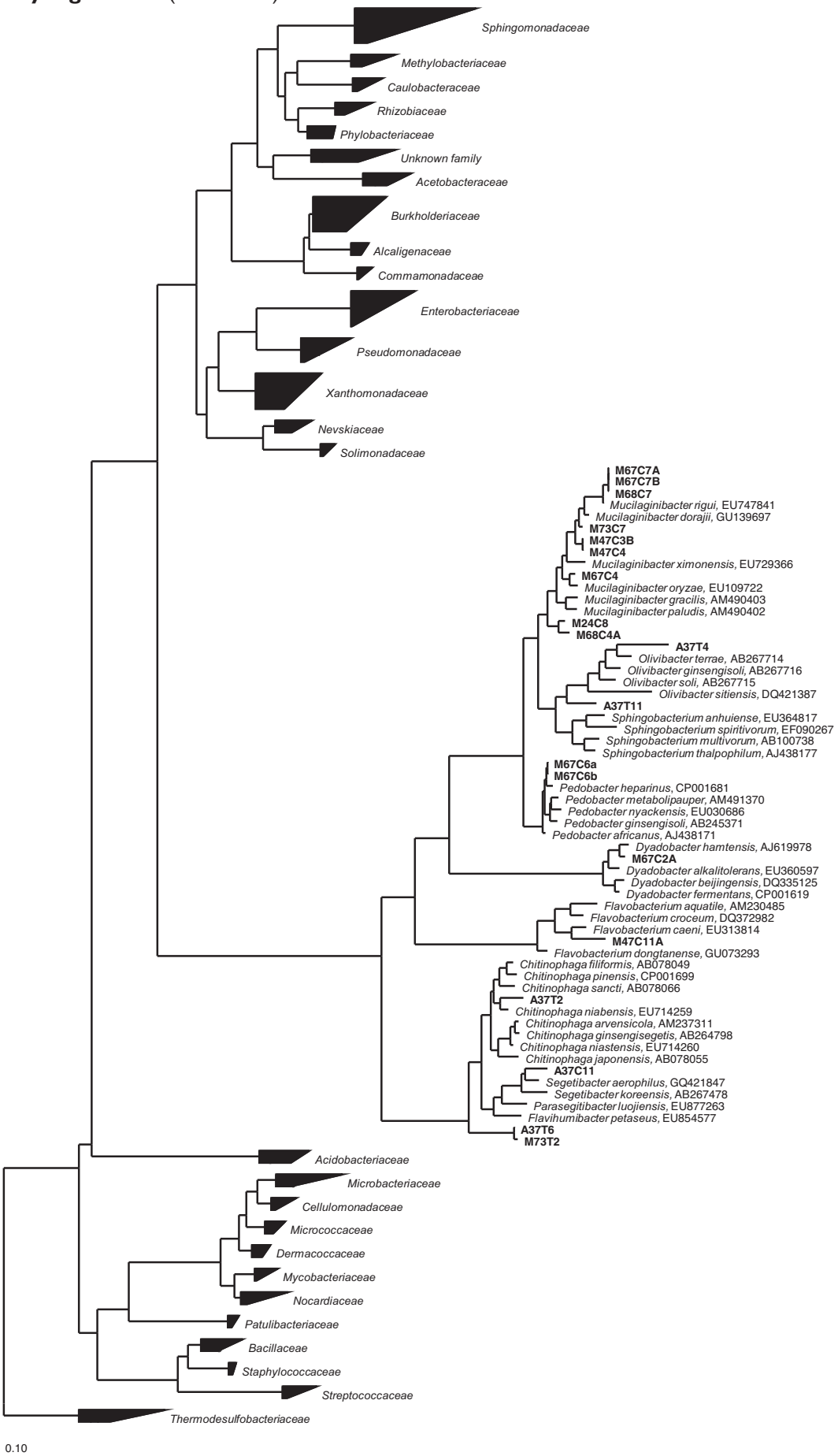

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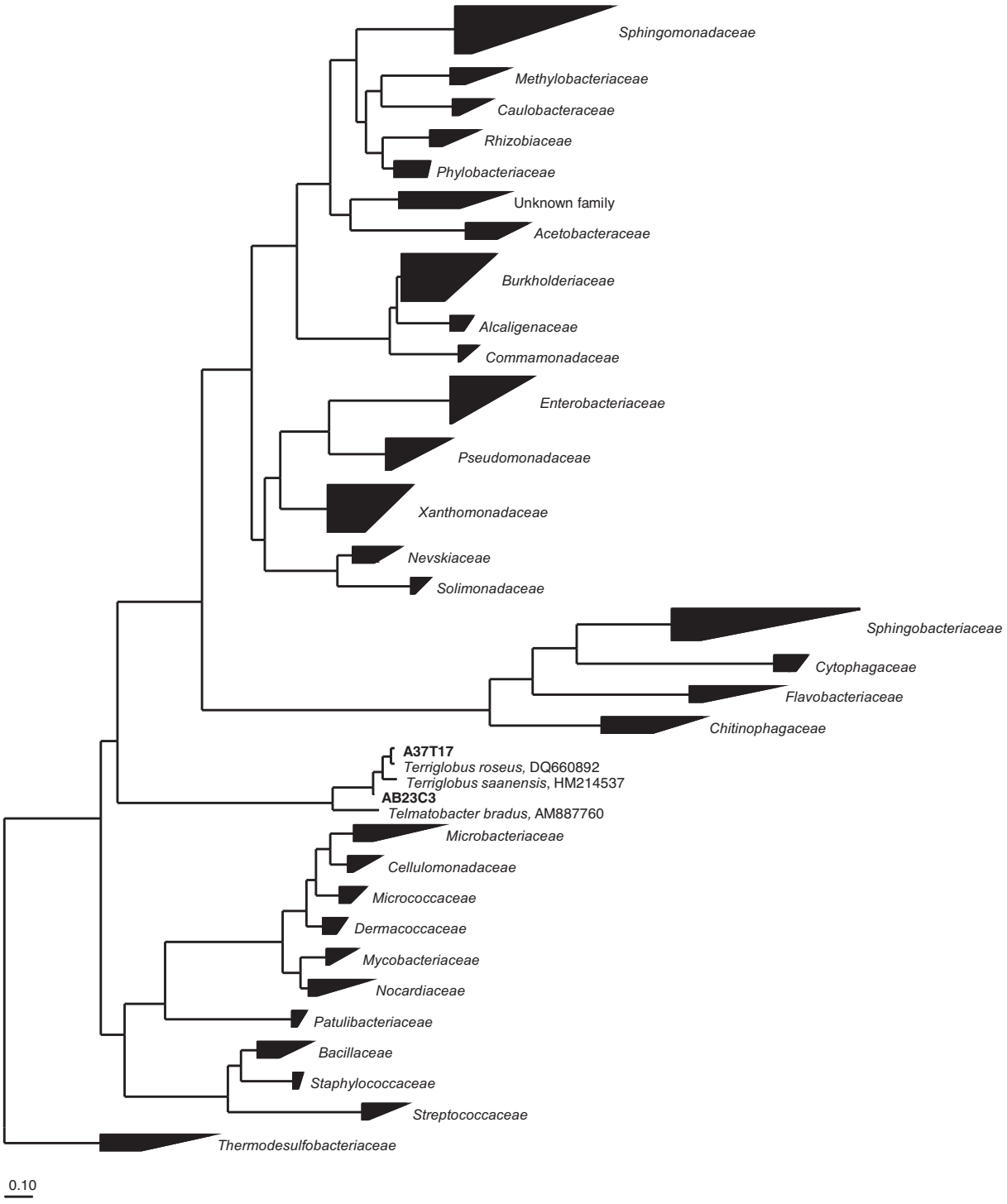

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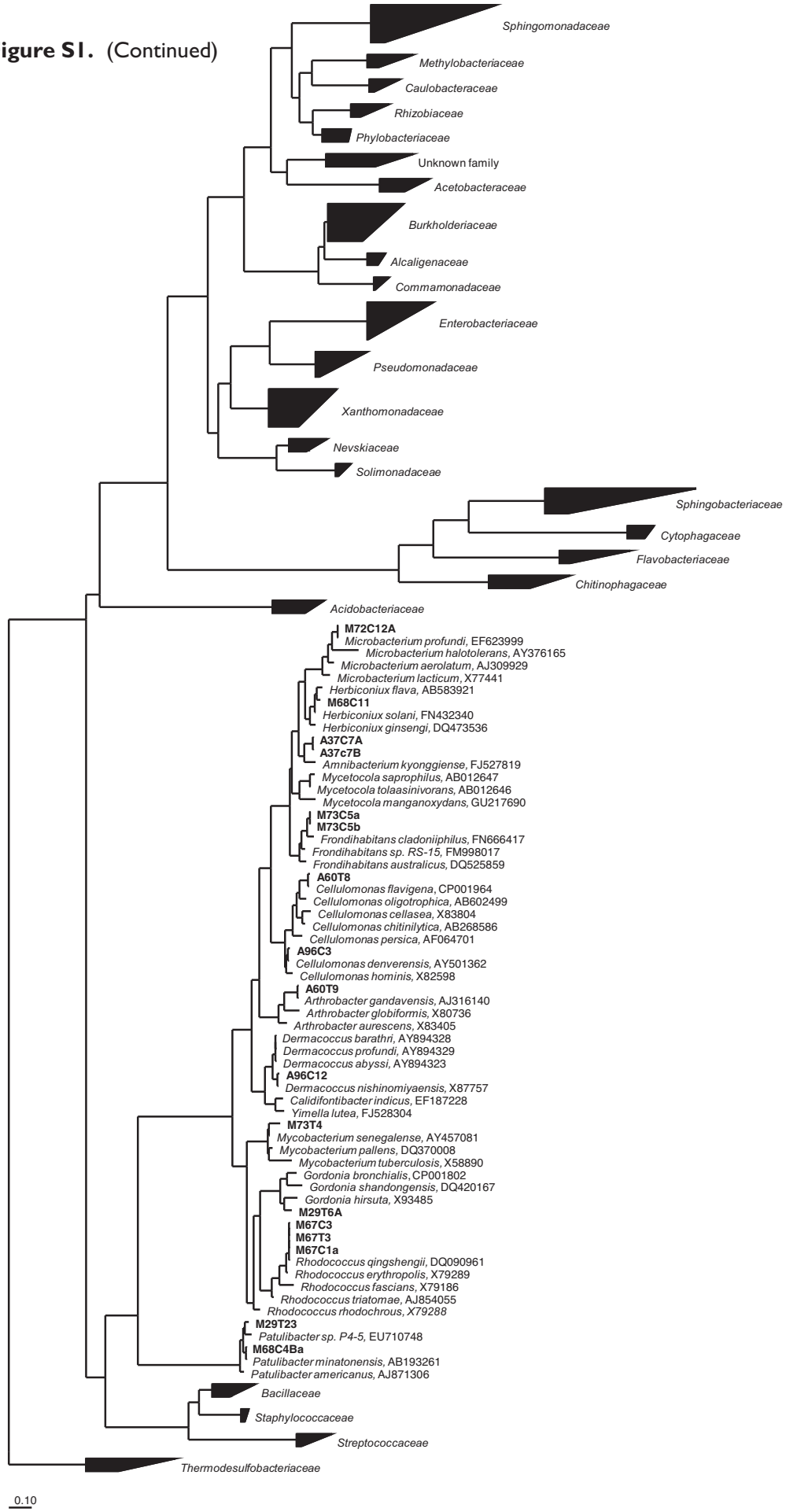

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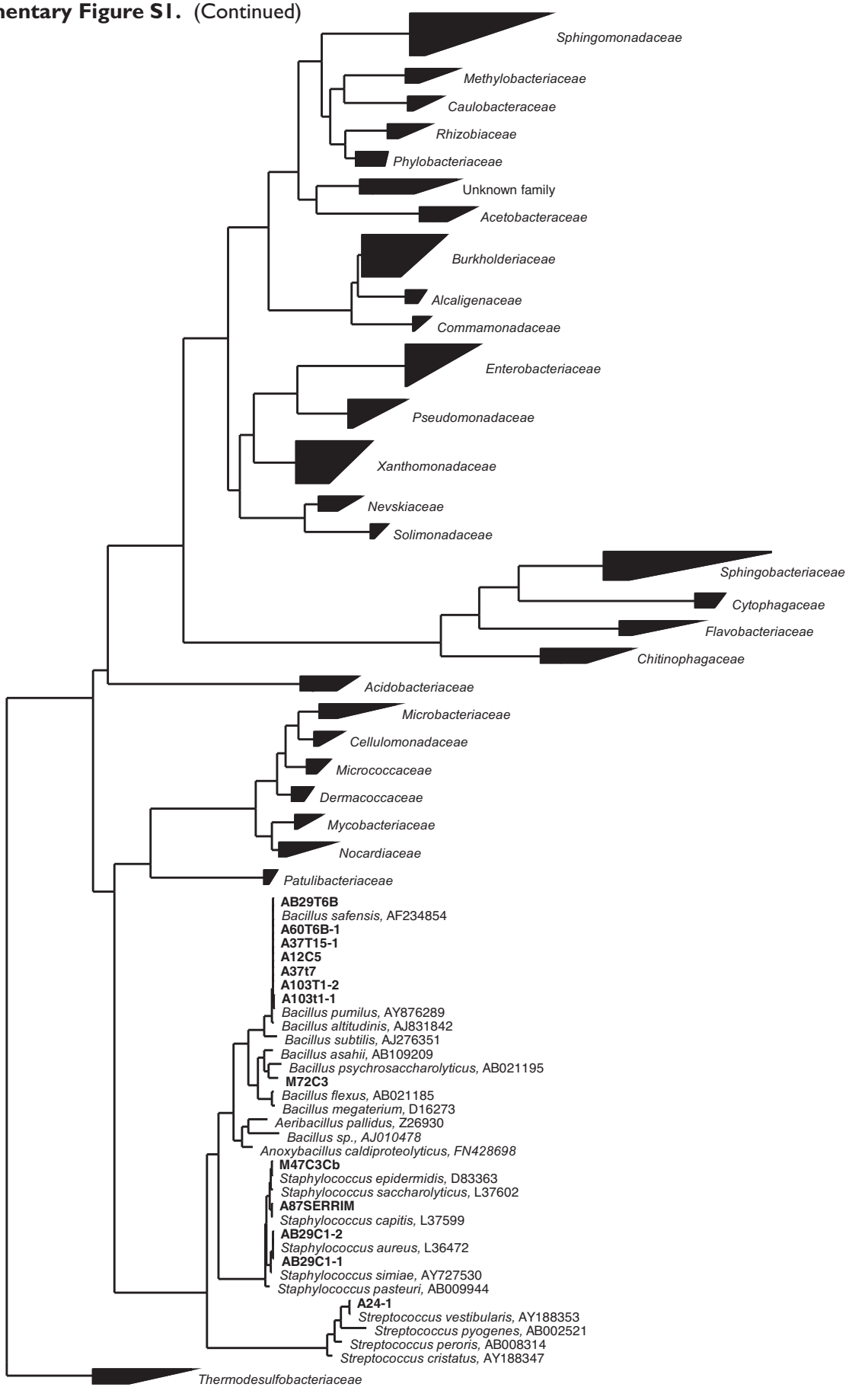
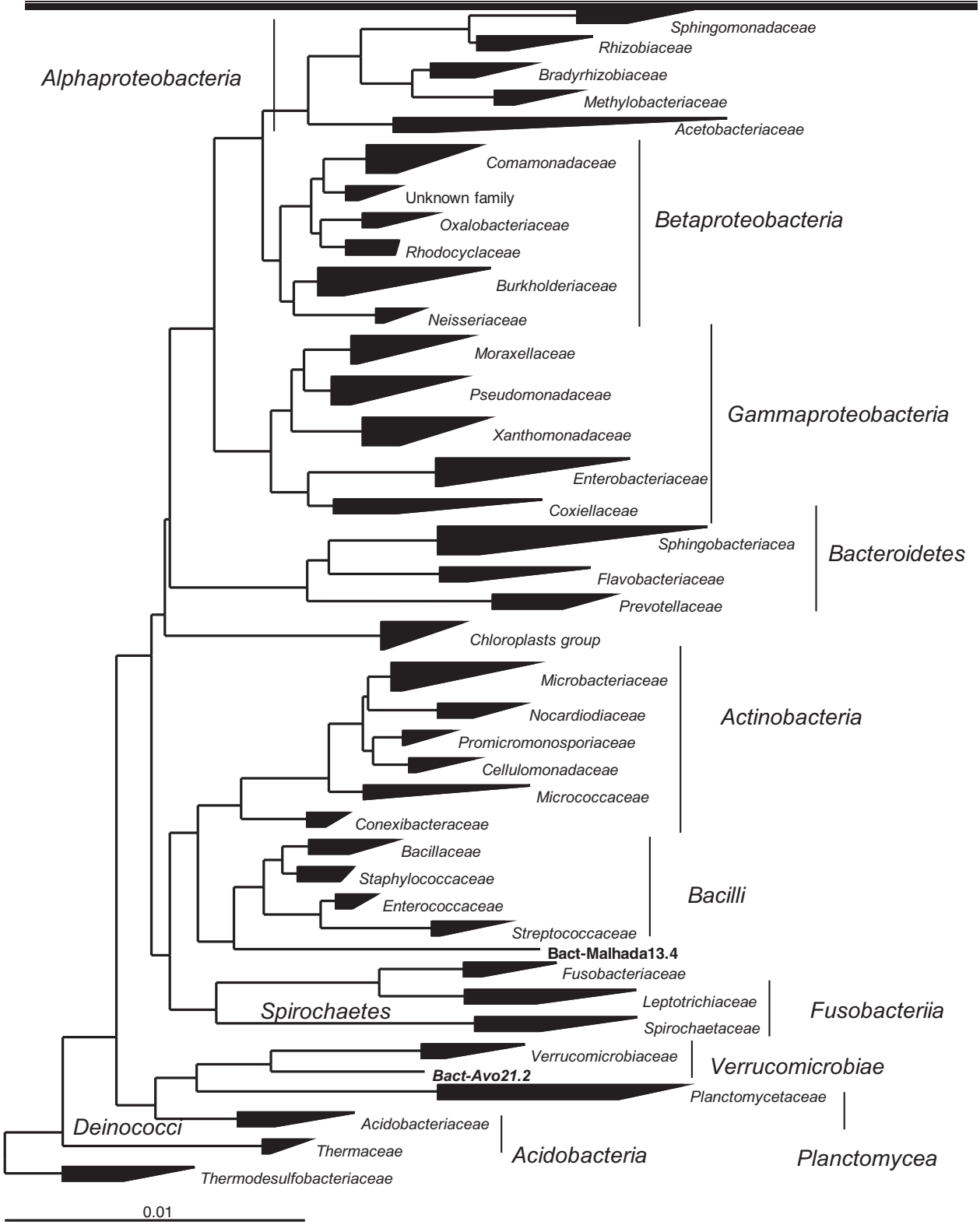


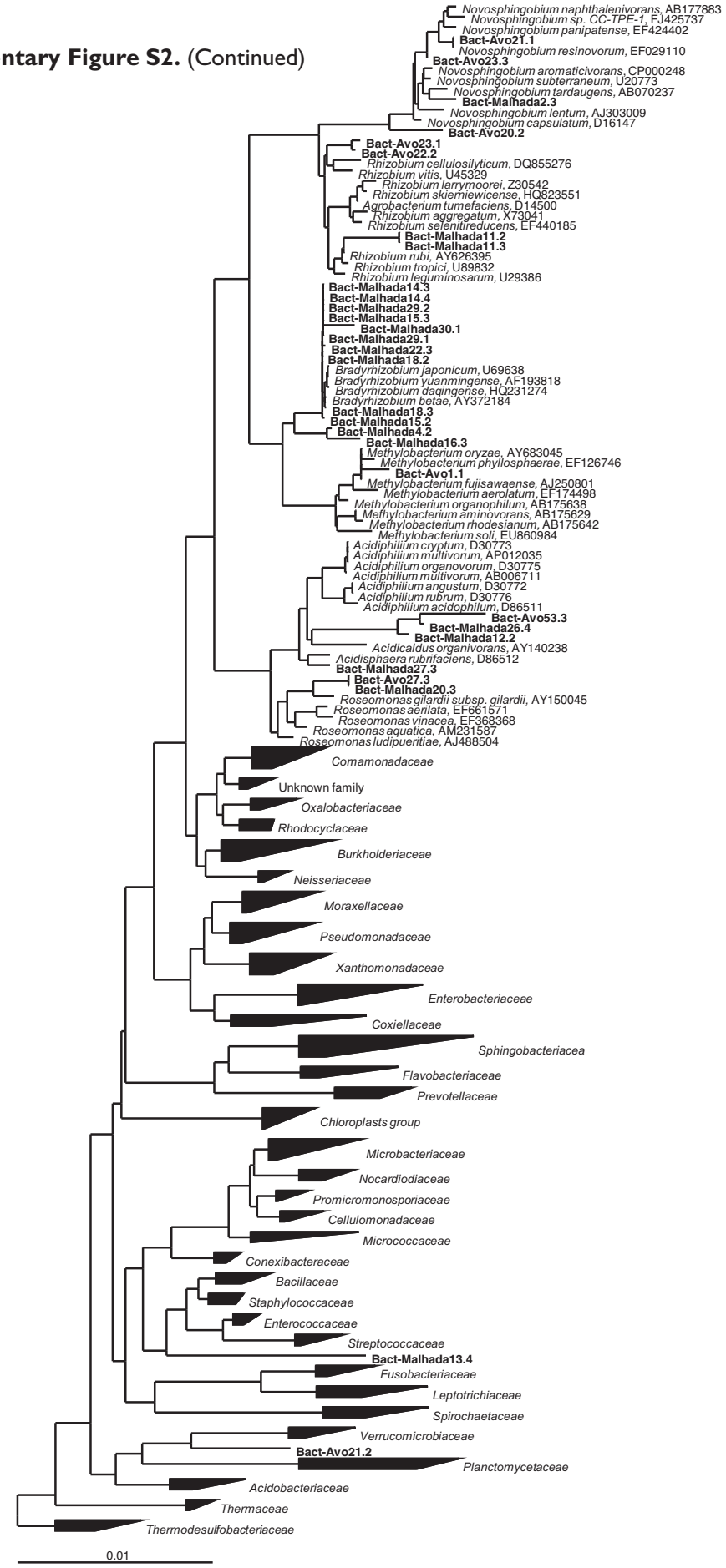
Appendix

B



Supplementary Figure S2. Phylogenetic analysis of bacterial 16S rRNA gene sequences of endophytic bacterial community by DGGE band clones analysis from pine trees. The dendrogram was constructed by the RAxML method with GTRGAMMA model included inside ARB software. The phylogenetic tree represents the classes *Alpha-, Beta-, Gammaproteobacteria, Actinobacteria, Bacilli, Fusobacteriia, Spirochaetes, Verrucomicrobiae, Planctomycea Acidobacteria, Deinococci,* a group of chloroplasts and the phylum *Bacteroidetes,* discriminates the families identified in this study and is rooted by the family *Thermodesulfobacteriaceae*. The following phylogenetic trees in next pages are the extension of these families clusters. The bacterial DGGE band clones in this study are indicated in bold, comprise the clones from areas Avô and Malhada (Bact-Avo and Bact-Malhada are clones from Avô and Malhada areas, respectively) and were compared with type strain of each species. Scale bar, 1 inferred nucleotide substitution per 100 nucleotides.

Supplementary Figure S2. (Continued)



Supplementary Figure S2. (Continued)

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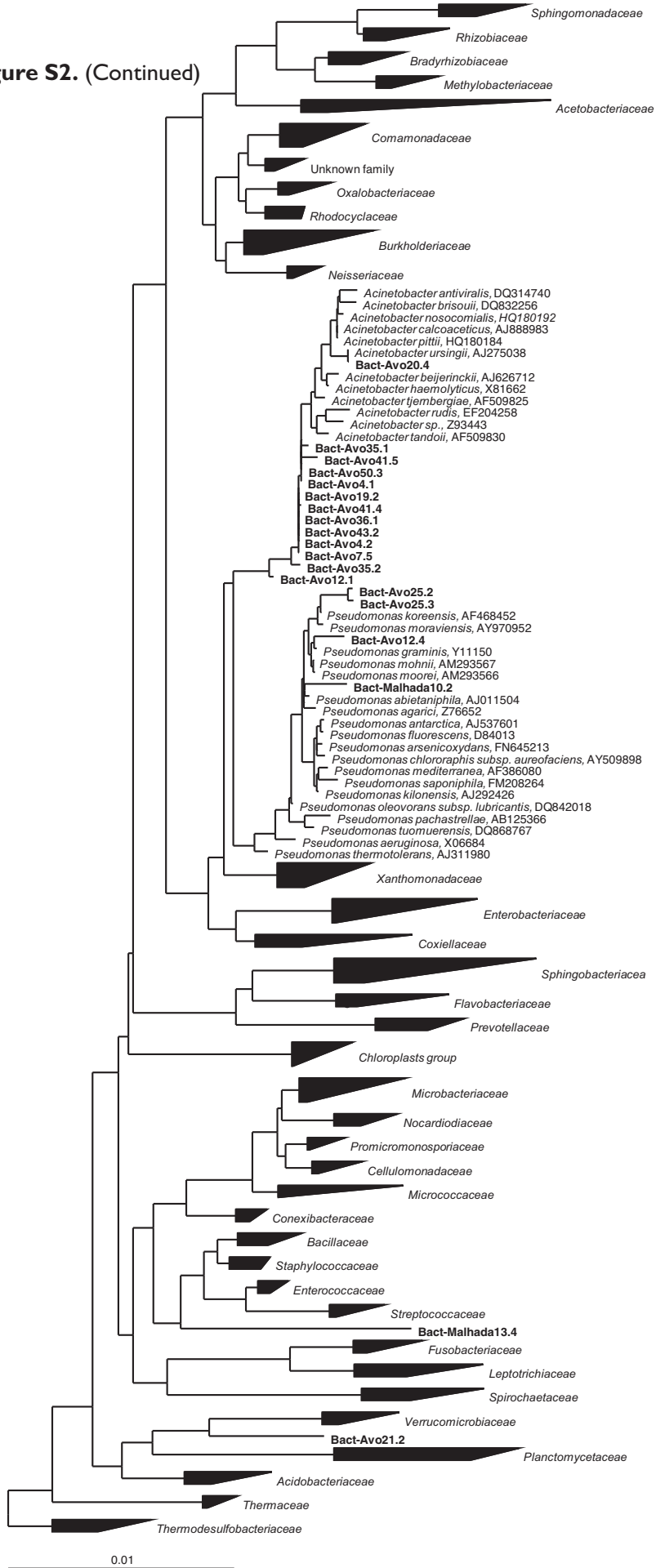

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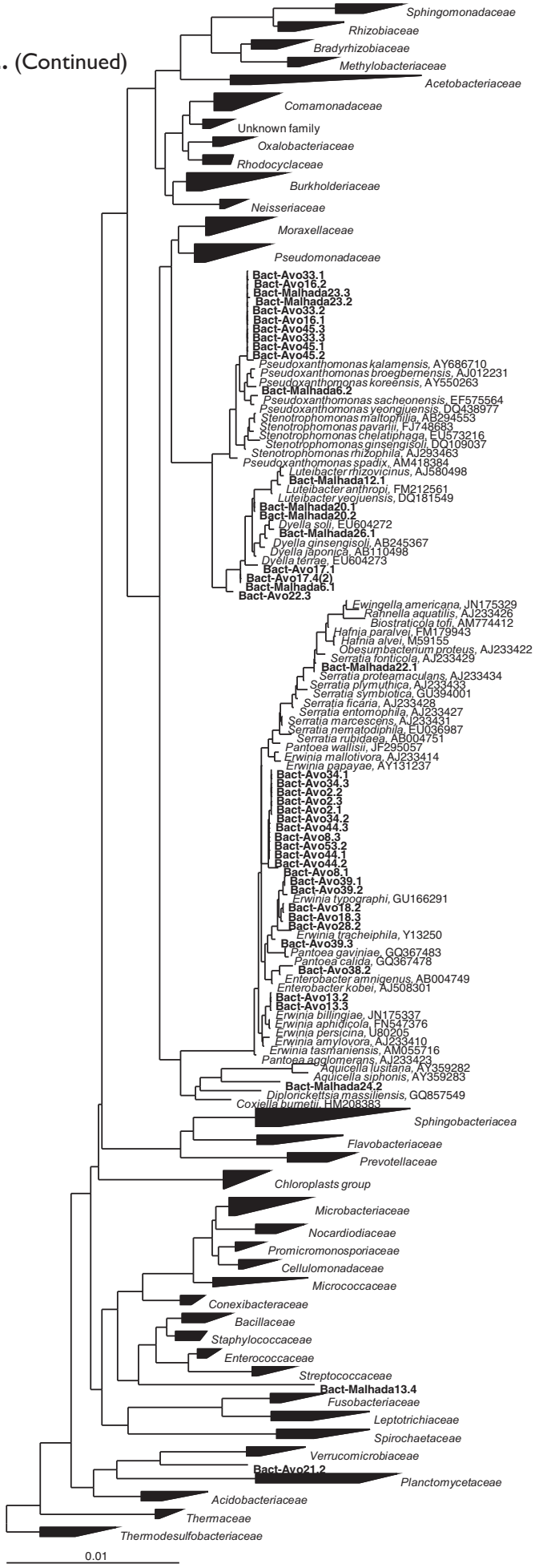

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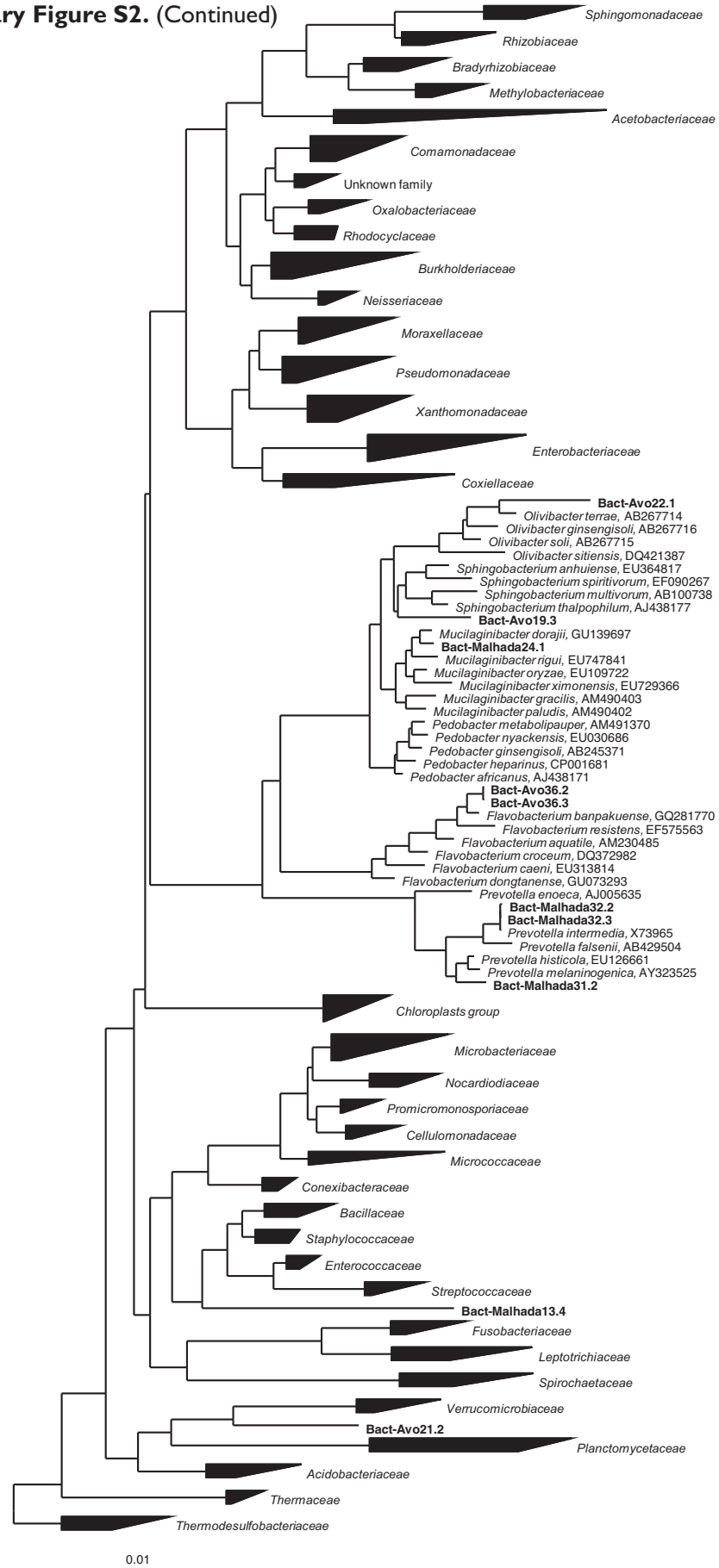

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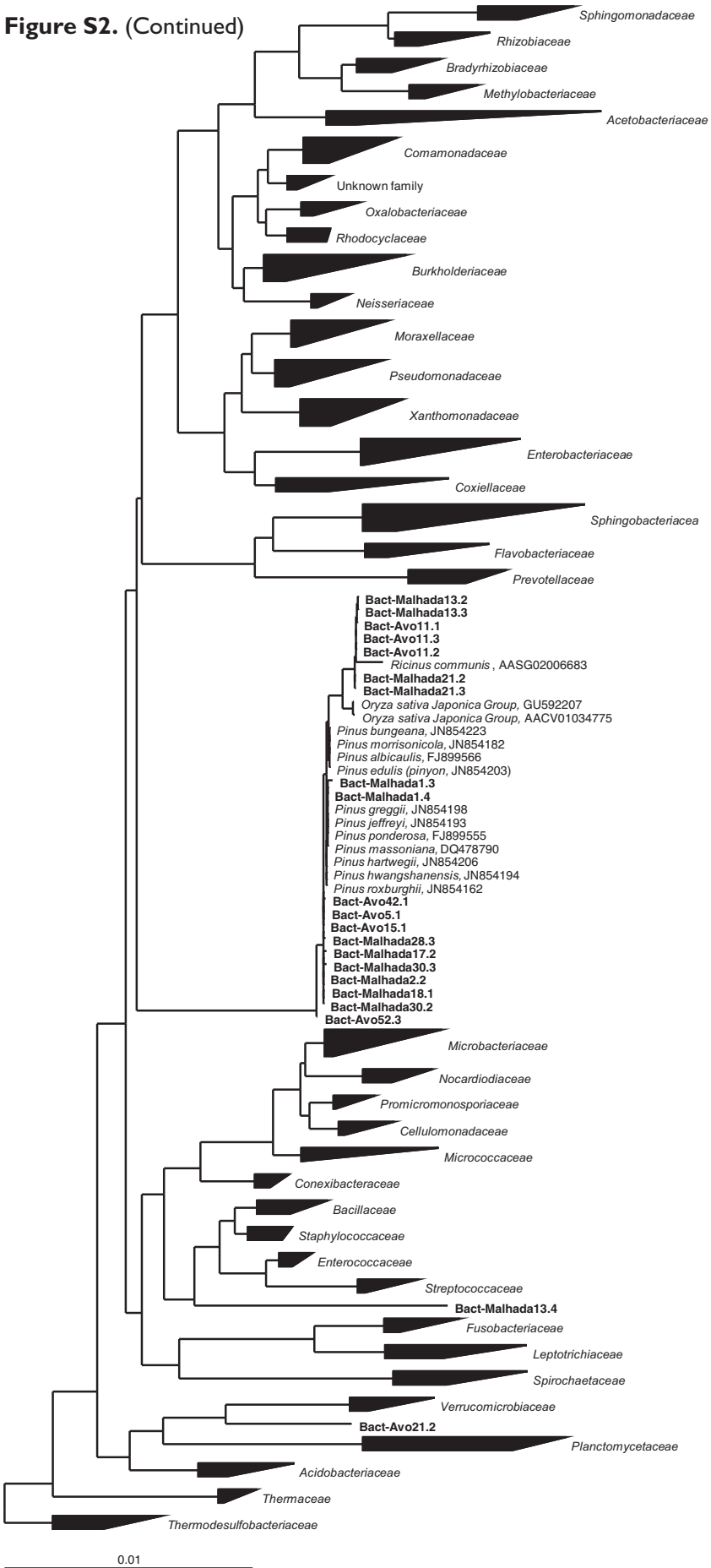

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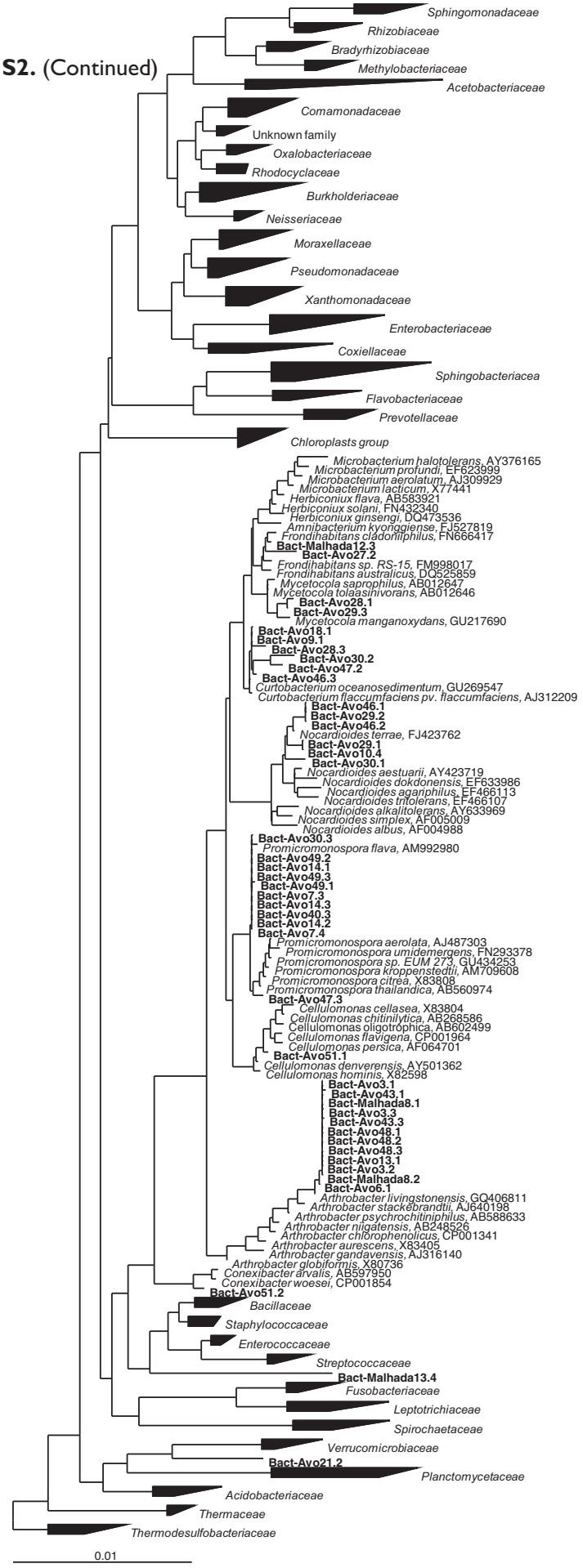

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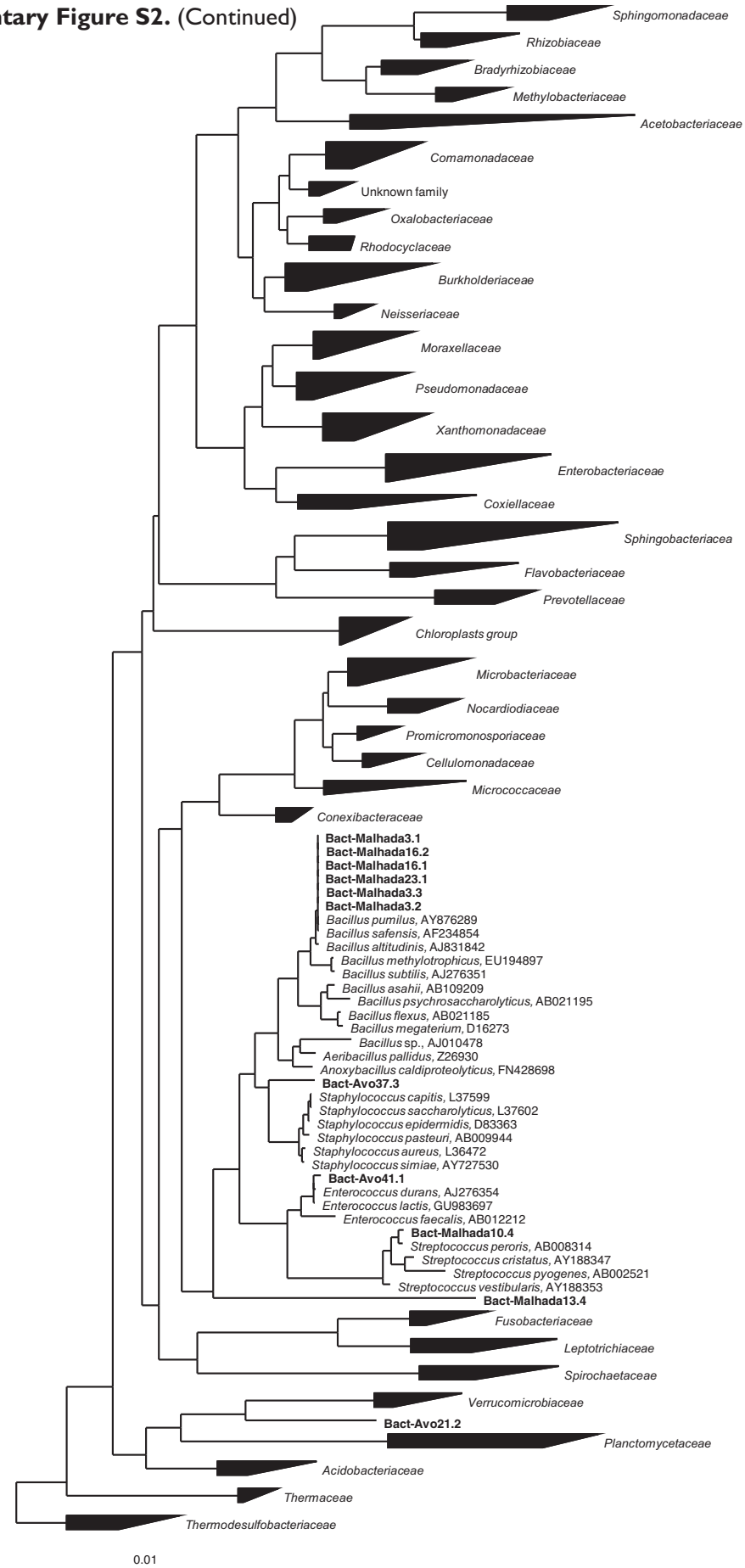

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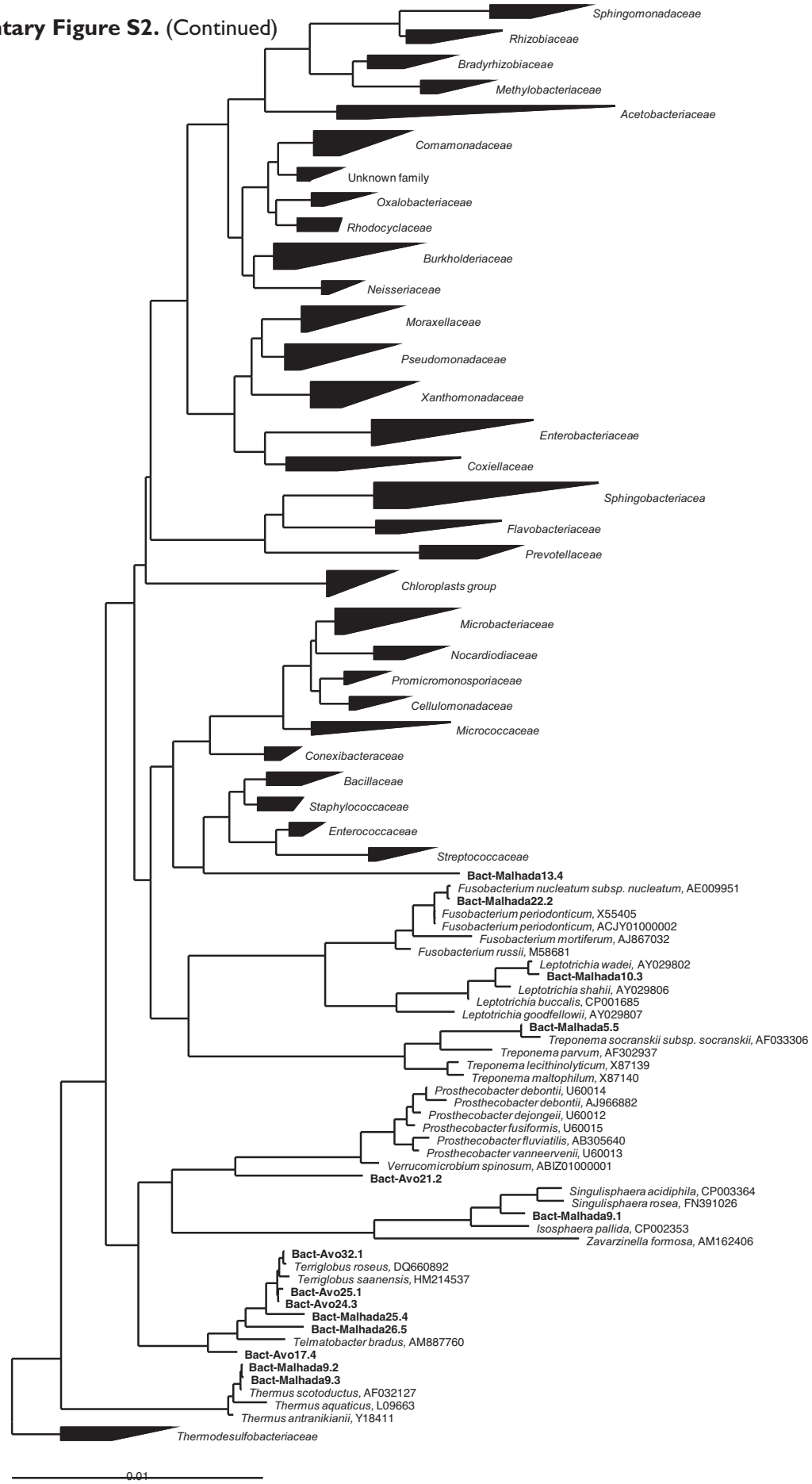
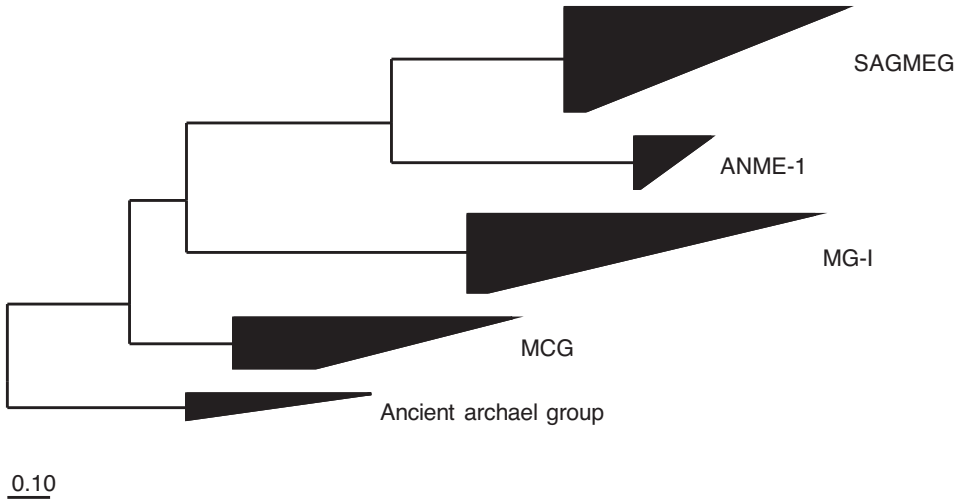
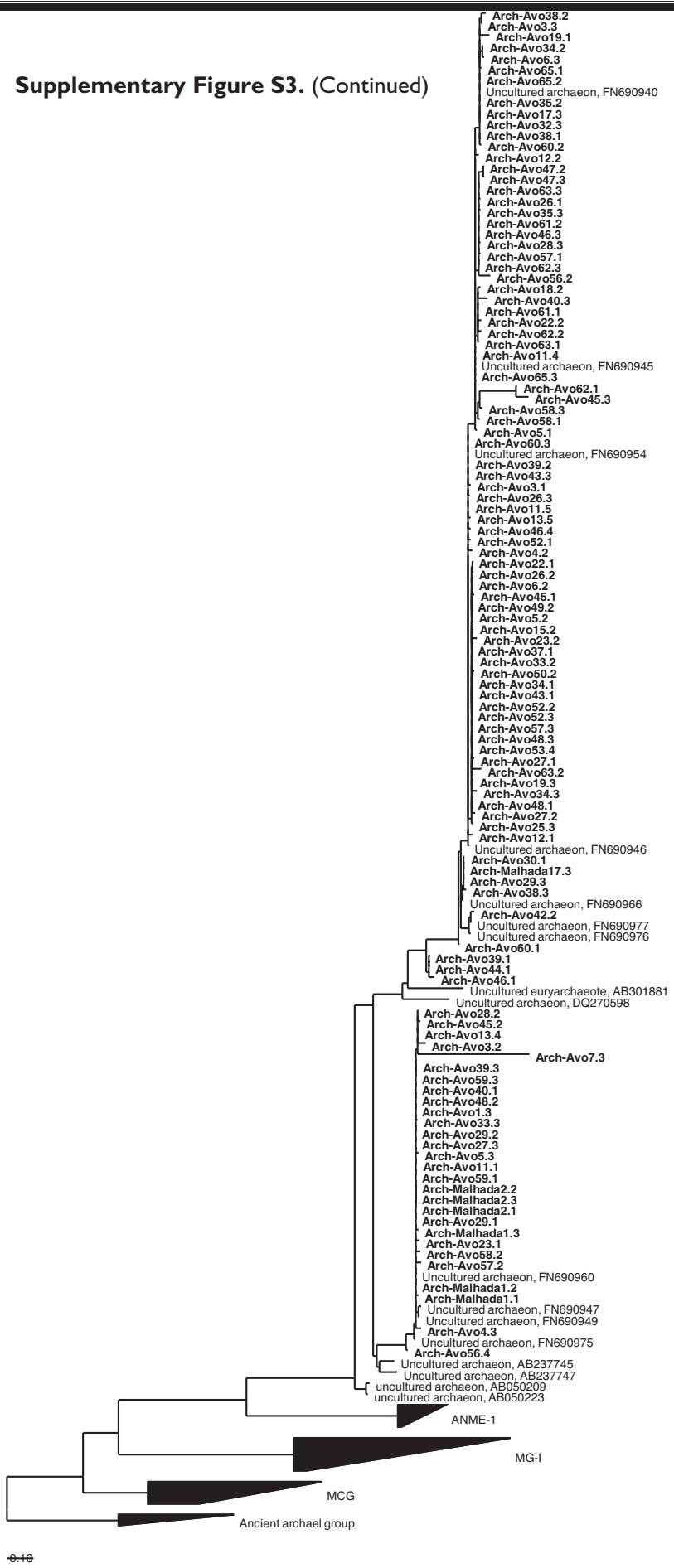


Appendix C

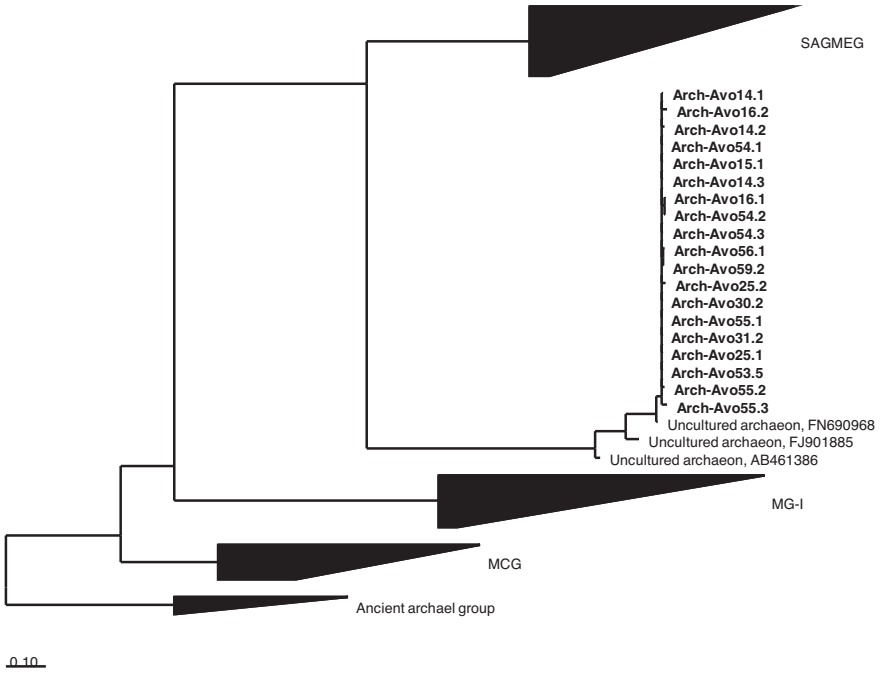


Supplementary Figure S3. Phylogenetic analysis of archaeal 16S rRNA gene sequences of endophytic archaeal community by DGGE band clones analysis from pine trees. The dendrogram was constructed by the RAxML method with GTRGAMMA model included inside ARB software. The phylogenetic tree represents the uncultured archaeal groups fro the phylum *Euryarchaeota* – uncultured archaea of the South African goldmine group (SAGMEG) and uncultured anaerobic methanotrophic archaea (ANME-1), the phylum *Thaumarchaeota* – uncultured marine group I (MG-I) and to the phylum *Crenarchaeota* – miscellaneous *Crenarchaeote* group (MCG), discriminates the groups identified in this study and is rooted by the Ancient archaeal group. The following phylogenetic trees in next pages are the extension of these group clusters. The archaeal DGGE band clones in this study are indicated in bold, comprise the clones from areas Avô and Malhada (Arch-Avo and Arch-Malhada are clones from Avô and Malhada areas, respectively) and were compared with uncultured and cultured archaea species. Scale bar, 10 inferred nucleotide substitution per 100 nucleotides.

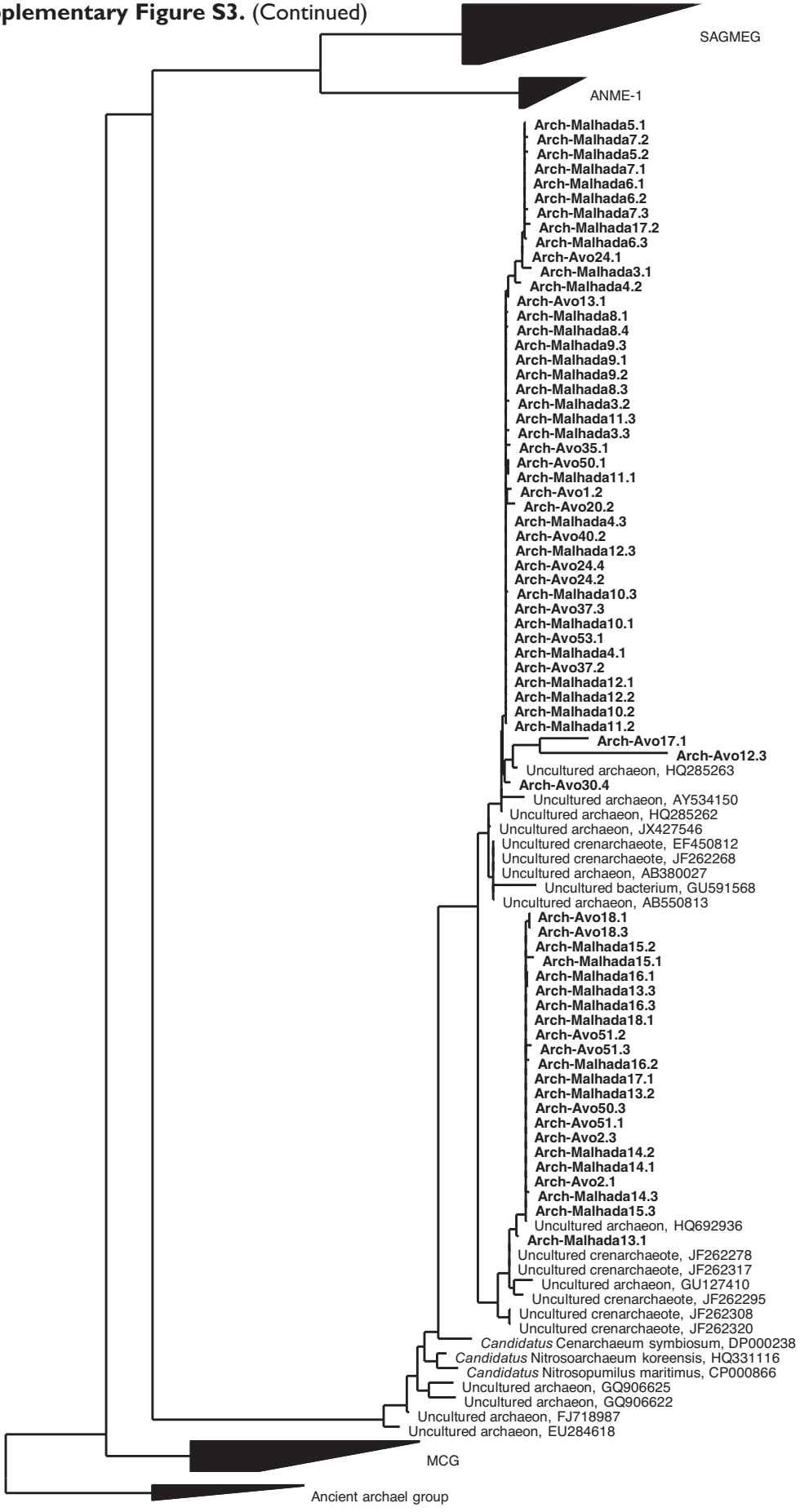
Supplementary Figure S3. (Continued)



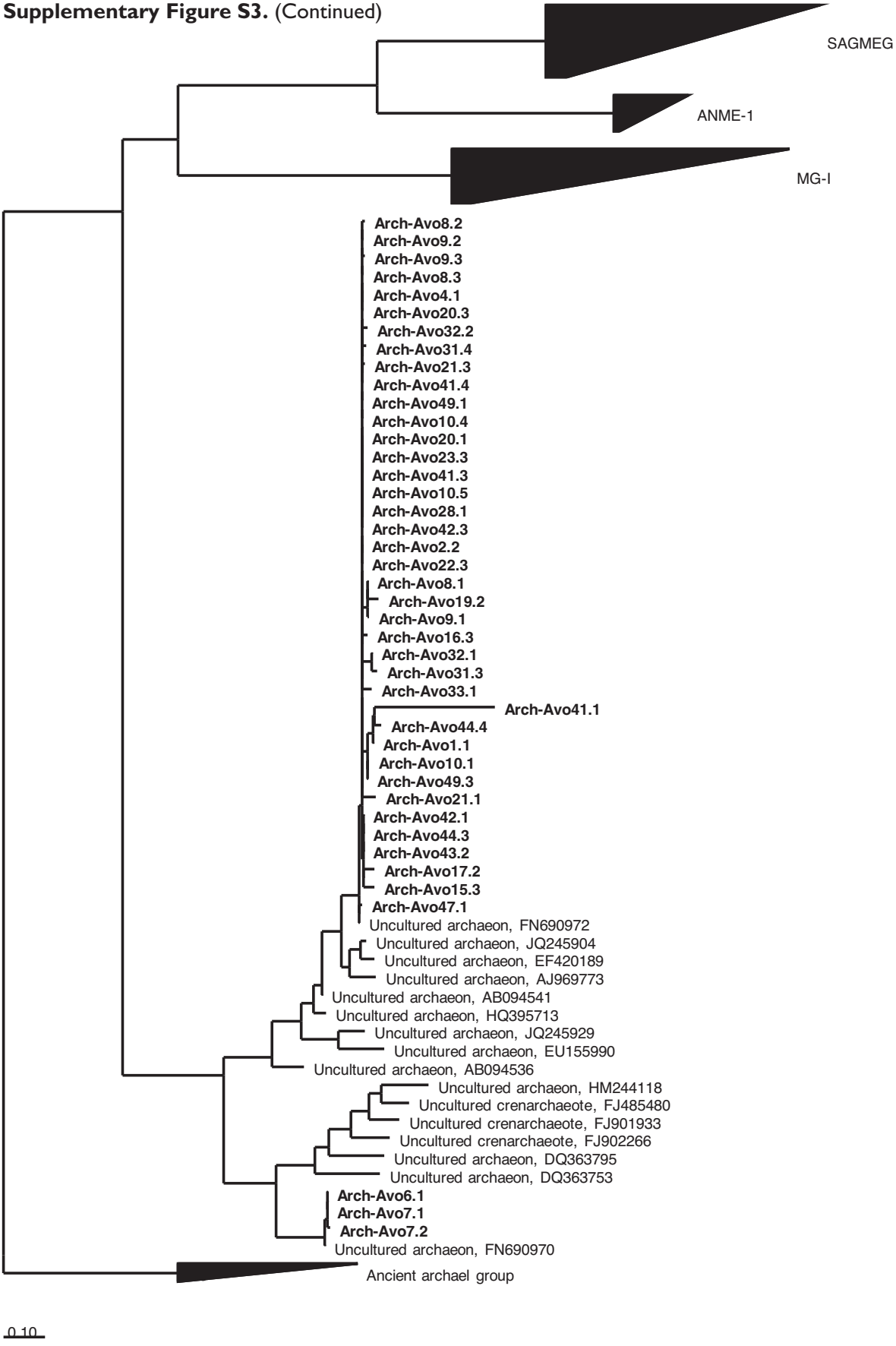
Supplementary Figure S3. (Continued)



Supplementary Figure S3. (Continued)



Supplementary Figure S3. (Continued)



Appendix

D

Supplementary Table S1 – Carbon source oxidation profile of strain A37T2^T and *C. niabensis* DSM 24787^T.

Strains: 1, A37T2^T (data from this study); 2, *C. niabensis* DSM 24787^T (data from this study). +, positive; w, weakly positive; –, negative. The remaining substrates not included in the table are not oxidized by both strains.

Carbon source (GN2 MicroPlates)	1	2
Dextrin	+	+
Tween 40	–	w
N-Acetyl-D-Galactosamine	–	+
N-Acetyl-D-Glucosamine	–	+
Adonitol	–	+
L-Arabinose	–	+
D-Cellobiose	+	+
D-Fructose	+	+
L-Fucose	–	+
D-Galactose	+	+
Gentiobiose	+	+
α-D-Glucose	+	+
α-D-Lactose	+	+
Lactulose	+	+
D-Maltose	+	+
D-Mannose	+	+
D-Melibiose	+	+
β-Methyl-D-Glucoside	+	+
D-Raffinose	–	+
L-Rhamnose	–	+
Sucrose	+	+
D-Trehalose	+	+
Turanose	+	+
Pyruvic Acid Methyl Ester	–	+
Succinic Acid Mono-Methyl-Ester	–	+
Acetic acid	–	w
D-Galacturonic acid	w	–
D-Gluconic acid	+	–
D-Glucuronic acid	–	+
α-Keto butyric acid	w	–
α-Keto valeric acid	–	w
D,L-Lactic acid	–	+
Sebatic acid	w	–
Succinic acid	–	+
Bromosuccinic acid	–	+
L-Alanine	–	w
L-Alanyl-glycine	–	+
L-Asparagine	–	+
L-Aspartic acid	w	+
L-Glutamic acid	w	w
Glycyl-L-aspartic acid	–	+
Glycyl-L-glutamic acid	–	w
L-Hydroxyproline	–	w
L-Proline	w	–
L-Serine	–	+
L-Threonine	w	–
Uridine	+	–
Thymidine	w	–
Phenylethylamine	w	–
Glycerol	–	+
D,L, α-Glycerol phosphate	–	+
α-D-Glucose-1-phosphate	+	+
D-Glucose-6-phosphate	+	w