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SHORT COMMUNICATION

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Pseudomonas associated with *Bursaphelenchus xylophilus*, its insect vector and the host tree: A role in pine wilt disease?

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Abstract

In this study, we characterized the diversity of *Pseudomonas* associated with *Bursaphelenchus xylophilus*, its insect vector (*Monochamus galloprovincialis*) and its host (*Pinus pinaster*), by a culture-independent approach using *rpoD* clone libraries. Clone libraries of *Pseudomonas rpoD* were obtained from *B. xylophilus*, *M. galloprovincialis* and infected *P. pinaster*. Most *M. galloprovincialis* and *B. xylophilus* sequences grouped together in the *P. fluorescens* group. Genes related to xenobiotics degradation and phenylacetate synthesis were present in the genomes of the type strains closely related to sequences retrieved from the nematode libraries. Results demonstrated that the nematode, during its life stages inside the tree, maintains a diverse *Pseudomonas* community that is closely related to the one associated with the insect vector. These bacteria might contribute to degradation of xenobiotics and tree weakening during the nematode tree infection.

KEYWORDS

Bursaphelenchus xylophilus, *Monochamus galloprovincialis*, pine wilt disease, *Pinus pinaster*, *Pseudomonas*

1 | INTRODUCTION

Pine wilt disease (PWD) is a devastating disease for coniferous forests in several countries in Europe and Asia. This disease is caused by the plant parasitic nematode *Bursaphelenchus xylophilus*. Short-distance spread between trees is mediated by cerambycid of the genus *Monochamus*. The nematodes are carried in the insect tracheae and enter a healthy tree through the feeding wound made by the insect (reviewed in Vicente, Espada, Vieira, & Mota, 2012).

In 1980, Oku and colleagues reported that a nematode-associated *Pseudomonas* sp. produced a toxin that contributed to tree wilting. *Pseudomonas* is one of the most abundant and frequently detected genera in association with *B. xylophilus*, proposed by several authors to establish a mutualistic symbiotic relationship with

the nematodes relevant in the PWD mechanism (reviewed by Nascimento, Hasegawa, Mota, & Vicente, 2014). However, the strategies previously applied were based on sequence analysis of the 16S rRNA gene, which does not give accurate intra-genus information (Mulet, Bennasar, Lalucat, & García-Valdes, 2009).

Therefore, the aim of this study was to unravel the diversity of *Pseudomonas* associated with PWD (the nematode, infected *Pinus pinaster* trees and insect vector), using *rpoD* clone libraries, to better understand the roles of this genus of bacteria in this disease. The gene *rpoD* is a single copy gene reported to provide superior resolution to assess *Pseudomonas* intra-genus diversity (Mulet et al., 2009).

2 | MATERIAL AND METHODS

2.1 | Samples and total bacterial DNA extraction

Samples were collected from infected *P. pinaster* trees in two PWD affected regions in Portugal: Góis (40°09′07.3″N 8°07′34.1″W) and Comporta (38°22′48.67″N/8°47′25.00″W). From each tree trunk, samples comprised sawdust, wood pieces for nematode extraction and logs for arising adult *M. galloprovincialis* as described in Alves et al. (2018). Total genomic DNA extraction was performed as described in Alves et al. (2018) on samples of nematodes, insects trachea (one insect trachea per sample) and sawdust (0.25 g per sample). A *B. xylophilus* molecular screening was performed in all samples as described in Alves et al. (2018), and only the positive ones were used. The DNA of 3 nematode samples, three insect samples and four sawdust samples was pooled for each species.

2.2 | Construction and analysis of the *Pseudomonas rpoD* clone libraries

Pseudomonas rpoD amplification was performed using the specific primers PsEG30F/ PsEG790R and cycle conditions described in Mulet et al. (2009). Clone libraries were constructed using the TA Cloning Kit, according to the manufacturer's instructions (Invitrogen) and *Escherichia coli* NZYStar competent cells (NZYTech). Clones were screened by PCR for the presence of fragments with the expected size. Amplicons were sequenced using GATC Biotech services. Sequences that were 100% identical were grouped to obtain a set of unique sequences (US). The obtained US are available in NCBI platform under the accession numbers MN379315-MN379435. Similarity searches in the GenBank database were performed using the BLAST tool against type strains after sequence editing. Phylogenetic trees were obtained in MEGA 7.0.

3 | RESULTS AND DISCUSSION

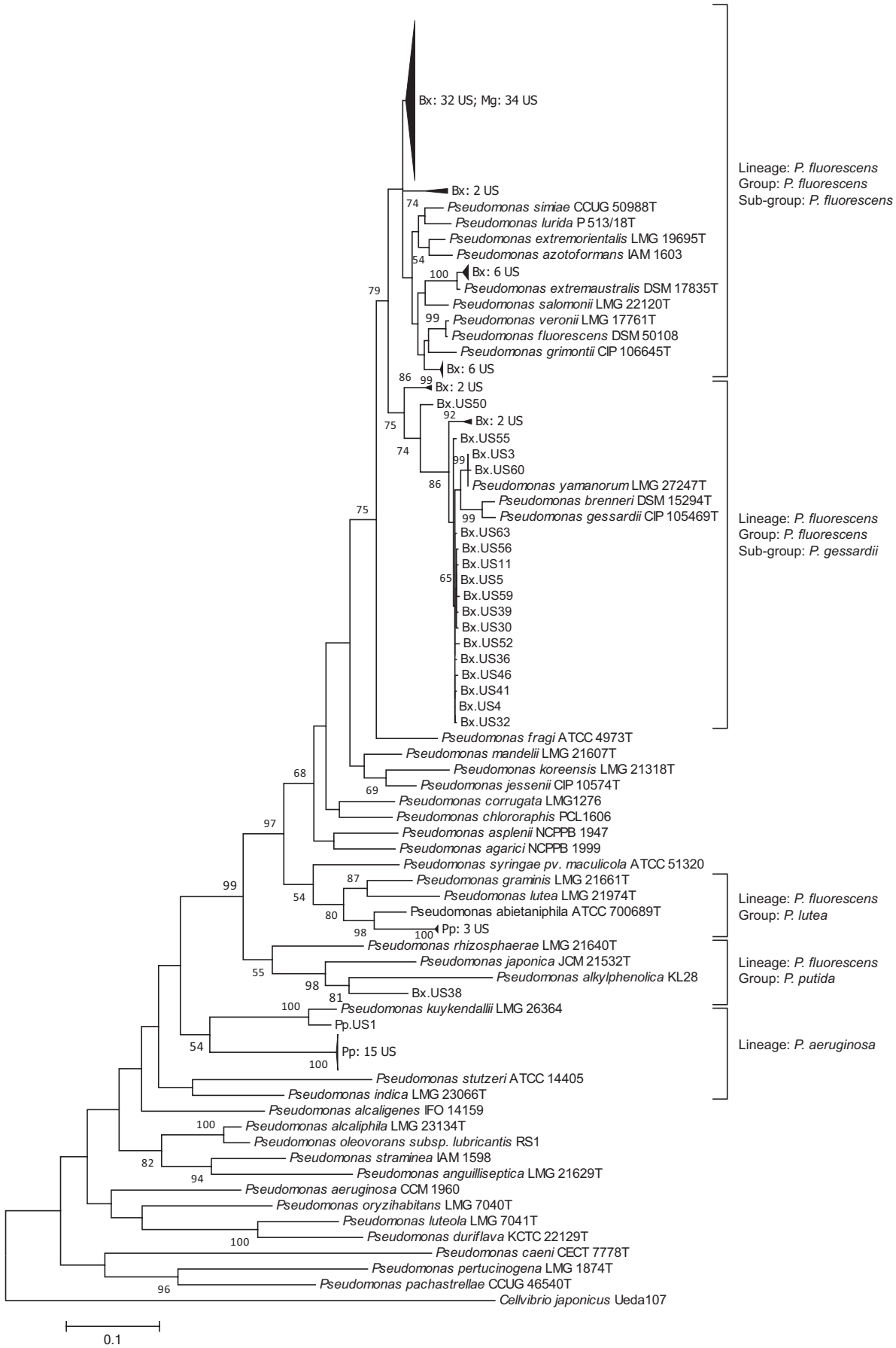
A total of 248 clones were obtained. BLAST analysis of the *rpoD* gene fragments [sizes between 704 and 710 pb after editing, corresponding to approximately 35% of the *rpoD* gene (around

TABLE 1 Summary of results obtained for clone libraries constructed for *Monochamus galloprovincialis*, *Bursaphelenchus xylophilus* and *Pinus pinaster* regarding the unique sequences (US) detected

		Number of US (clones)[b]		
Closest relative type strains	Similarity (%)[a]	*M. galloprovincialis*	*B. xylophilus*	*P. pinaster*
Pseudomonas extremorientalis LMG 19695T	95/95–96	34 (74)	33 (48)	0
Pseudomonas yamanorum LMG 27247T	94–100	0	20 (28)	0
Pseudomonas extremaustralis DMS 17835T	100	0	6 (8)	0
Pseudomonas veronii LMG 17761T	95–96	0	6 (13)	0
Pseudomonas grimontii CIP 106645T	94	0	1 (1)	0
Pseudomonas japonica JMC 21532T	84	0	1 (1)	0
Pseudomonas kuykendallii LMG 26364T	80–95	0	0	16 (23)
Pseudomonas abietaniphila ATCC 700689T	90–100	0	0	3 (3)
Pseudomonas salomonii LMG 22120T	94	0	1 (1)	0
Total number of US (clones)		34 (74)	68 (100)	19 (26)
% identity between *rpoD* sequences		98.9%–100%	77%–100%	71.2%–100%

[a]Percentage of similarity between the obtained *rpoD* sequences of each clone library and the sequence of the closest relative type strain retrieved from BLAST.
[b]Number of US (clones) for each clone library similar to the respective closest type strains.

FIGURE 1 Maximum-likelihood tree illustrating the phylogenetic position of the *Pseudomonas rpoD* unique sequences (US) obtained in this study, the closest related *Pseudomonas* type strains and *rpoD* sequences from *Pseudomonas* strains from each *Pseudomonas* group according to Mulet et al. (2012) and Peix et al. (2018). The *Cellvibrio japonicus* Ueda107 *rpoD* (CP000934.1) sequence was used as out-group. Bootstrap values of more than 500 (from 1,000 replicates) are indicated at the nodes. The number of US in each compressed subtree is indicated for each species (Bx—*Bursaphelenchus xylophilus*; Mg—*Monochamus galloprovincialis*; Pp—*Pinus pinaster*)


Bx: 32 US; Mg: 34 US
Bx: 2 US
Pseudomonas simiae CCUG 50988T
Pseudomonas lurida P 513/18T
Pseudomonas extremorientalis LMG 19695T
Pseudomonas azotoformans IAM 1603
Bx: 6 US
Pseudomonas extremaustralis DSM 17835T
Pseudomonas salomonii LMG 22120T
Pseudomonas veronii LMG 17761T
Pseudomonas fluorescens DSM 50108
Pseudomonas grimontii CIP 106645T
Bx: 6 US
Bx: 2 US
Bx.US50
Bx: 2 US
Bx.US55
Bx.US3
Bx.US60
Pseudomonas yamanorum LMG 27247T
Pseudomonas brenneri DSM 15294T
Pseudomonas gessardii CIP 105469T
Bx.US63
Bx.US56
Bx.US11
Bx.US5
Bx.US59
Bx.US39
Bx.US30
Bx.US52
Bx.US36
Bx.US46
Bx.US41
Bx.US4
Bx.US32
Pseudomonas fragi ATCC 4973T
Pseudomonas mandelii LMG 21607T
Pseudomonas koreensis LMG 21318T
Pseudomonas jessenii CIP 10574T
Pseudomonas corrugata LMG1276
Pseudomonas chlororaphis PCL1606
Pseudomonas asplenii NCPPB 1947
Pseudomonas agarici NCPPB 1999
Pseudomonas syringae pv. maculicola ATCC 51320
Pseudomonas graminis LMG 21661T
Pseudomonas lutea LMG 21974T
Pseudomonas abietaniphila ATCC 700689T
Pp: 3 US
Pseudomonas rhizosphaerae LMG 21640T
Pseudomonas japonica JCM 21532T
Pseudomonas alkylphenolica KL28
Bx.US38
Pseudomonas kuykendallii LMG 26364
Pp.US1
Pp: 15 US
Pseudomonas stutzeri ATCC 14405
Pseudomonas indica LMG 23066T
Pseudomonas alcaligenes IFO 14159
Pseudomonas alcaliphila LMG 23134T
Pseudomonas oleovorans subsp. lubricantis RS1
Pseudomonas straminea IAM 1598
Pseudomonas anguilliseptica LMG 21629T
Pseudomonas aeruginosa CCM 1960
Pseudomonas oryzihabitans LMG 7040T
Pseudomonas luteola LMG 7041T
Pseudomonas duriflava KCTC 22129T
Pseudomonas caeni CECT 7778T
Pseudomonas pertucinogena LMG 1874T
Pseudomonas pachastrellae CCUG 46540T
Cellvibrio japonicus Ueda107
0.1
Lineage: P. fluorescens
Group: P. fluorescens
Sub-group: P. fluorescens
Lineage: P. fluorescens
Group: P. fluorescens
Sub-group: P. gessardii
Lineage: P. fluorescens
Group: P. lutea
Lineage: P. fluorescens
Group: P. putida
Lineage: P. aeruginosa

1850pb)] revealed 202 clones with similarities ranging from 84% to 100% to *Pseudomonas* spp. type strains, 44 clones with similarities ranging from 85% to 88% to *Dyella thiooxydans* ATSB10^T and two clones with 99% similarities to *Serratia ficaria* NCTC 12148^T. The 46 clones that affiliated with *Dyella* and *Serratia* belonged to the *P. pinaster* clone library. Sequences affiliating with other bacterial genera (e.g., *Alcalinovorax*) were occasionally amplified with these primers in previous studies (Mulet et al., 2009). After discarding sequences that did not affiliate with *Pseudomonas*, 26 clones remained for *P. pinaster* corresponding to 19 US (named Pp.US1-19), 100 for *B. xylophilus* that corresponded to 68 US (named Bx.US1-68) and 74 clones *for M. galloprovincialis* (named Mg.US1-74) that corresponded to 34 US. The BLAST analysis against *Pseudomonas* type strains is listed in Table 1. The phylogenetic tree (Figure 1) was constructed based on the maximum-likelihood method using the *rpoD* US from the clone libraries, the closest related type strains listed in Table 1 and strains representative of each group and subgroup of the *Pseudomonas* genus according to Mulet et al. (2012) and Peix, Ramírez-Bahena, and Velázquez (2018). Affiliation of the isolates was based on well-supported monospecific clades (bootstrap values above 50) in the phylogenetic tree obtained. Most of the retrieved sequences for *B. xylophilus* affiliated with the *Pseudomonas fluorescens* group (Mulet et al., 2012; Peix et al., 2018) together with all the *M. galloprovincialis* US. Moreover, the sequences Mg.US8 (comprising 33 clones) and Bx.US7 (three clones) were 100% identical. *P. pinaster* US affiliated with the *P. lutea* group (three US) and *P. aeruginosa* lineage (16 US), thus representing a different *Pseudomonas* community from that observed for the nematode and the insect vector.

Although *B. xylophilus* was collected from the tree and not from the insect, its associated *Pseudomonas* community was closely related to the one associated with the insect vector.

Bursaphelenchus xylophilus sequences affiliated with *P. alkylphenolica* as well as with *P. yamanorum* and *P. extremaustralis*. In the genomes of *P. yamanorum* (accession number LT629793) and *P. extremaustralis* strains (accession numbers FUYI00000000.1, LT629689 and AHIP00000000.1), we found genes coding for enzymes for the complete degradation of benzoate, a common intermediate in the anaerobic metabolism of toxic compounds, like phenols and other aromatic metabolites produced by the tree as a defence mechanism. Moreover, genes coding for enzymes that could help in nematode protection against oxidative stress were also detected in *P. yamanorum* and *P. extremaustralis* genomes (e.g., genes coding for catalases, peroxidases, redox proteins, glutathione S-transferase). The genomes of the four *Pseudomonas* strains had genes coding for the necessary enzymes for phenylacetate production. This compound was previously described as being produced by nematode-associated bacteria causing wilting symptoms (Proença, Grass, & Morais, 2016).

This is the first study analysing the diversity of *Pseudomonas* associated with PWD. The shared phylotypes between the nematode and the insect vector support the hypothesis that the nematode inherits part of its microbiome from the insect vector keeping it in its life stage inside the tree. The *Pseudomonas* strains detected may help the nematode in the degradation of xenobiotic compounds found inside the tree host and in tree weakening.

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