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Research Article

RESEARCH ARTICLE

The role of bacteria in pine wilt disease: insights from microbiome analysis

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One sentence summary: Bacteria play an important role in pine wilt disease development and may be targets for biological control.

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ABSTRACT

Pine Wilt Disease (PWD) has a significant impact on Eurasia pine forests. The microbiome of the nematode (the primary cause of the disease), its insect vector, and the host tree may be relevant for the disease mechanism. The aim of this study was to characterize these microbiomes, from three PWD-affected areas in Portugal, using Denaturing Gradient Gel Electrophoresis, 16S rRNA gene pyrosequencing, and a functional inference-based approach (PICRUSt). The bacterial community structure of the nematode was significantly different from the infected trees but closely related to the insect vector, supporting the hypothesis that the nematode microbiome might be in part inherited from the insect. Sampling location influenced mostly the tree microbiome ($P < 0.05$). Genes related both with plant growth promotion and phytopathogenicity were predicted for the tree microbiome. Xenobiotic degradation functions were predicted in the nematode and insect microbiomes. Phytotoxin biosynthesis was also predicted for the nematode microbiome, supporting the theory of a direct contribution of the microbiome to tree-wilting. This is the first study that simultaneously characterized the nematode, tree and insect-vector microbiomes from the same affected areas, and overall the results support the hypothesis that the PWD microbiome plays an important role in the disease's development.

Keywords: microbiome; next-generation sequencing; pine wilt disease; *Bursaphelenchus xylophilus*; *Pinus pinaster*; *Monochamus galloprovincialis*

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INTRODUCTION

Pine Wilt Disease (PWD) affects coniferous trees worldwide with far-reaching economic and environmental impacts (Mota and Vieira 2008; Futai 2013; Sousa, Vale and Abrantes 2015). *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), the pinewood nematode (PWN), is the only recognized causative agent of the disease and considered an A1 quarantine pest in the European Union (EPPO 2013, 2014). The PWN feeds on epithelial cells and living parenchyma of the tree, spreading and multiplying in the vascular system and resin canals, and ultimately obstructs water conductance. An infected tree can die within weeks following nematode infection (Zhao *et al.* 2014). The natural transmission of *B. xylophilus* from tree to tree depends on its insect vector, a sapro-xylophagous beetle of the genus *Monochamus* (Cerambycidae: Coleoptera) (Sousa *et al.* 2001). *Monochamus* insects oviposit in the trees and the PWN, in its dispersal stage, is attracted to the insects' pupal chambers. Once inside the chambers, nematodes enter the insect vector (callow adult stage) and concentrate mainly within the host trachea (Kobayashi, Yamane and Ikeda 1984; Naves, Bonifácio and Sousa 2016). After the insect's emergence from the tree for feeding and/or oviposition, the nematode will leave the insect body and enter another tree host (Jones *et al.* 2008).

The native range of *B. xylophilus* is within North America, where it is harmful only to the exotic coniferous trees (Mota and Vieira 2008). In Asia and Europe, *B. xylophilus* became a major pest due to wood transportation and the introduction of PWN-contaminated wood (Vicente *et al.* 2012a). In Europe it was first detected in Portugal (Setúbal Peninsula, 30 km south of Lisbon) in 1999 (Mota *et al.* 1999). In Portugal, the identified hosts are *Pinus pinaster* and *Pinus nigra* (Inácio *et al.* 2014) and the only known insect vector is *Monochamus galloprovincialis* (Mota *et al.* 1999; Sousa *et al.* 2001). Despite efforts to constrain the disease, it has spread to other regions, having been detected in Madeira Island in 2010 (Fonseca *et al.* 2012) and in north-western Spain (Abelleria *et al.* 2011).

B. xylophilus genomics and transcriptomics have unveiled several parasitism genes important for cell wall degradation, evasion of the host immune response, and detoxification processes (Kikuchi *et al.* 2011; Espada *et al.* 2015; Cardoso *et al.* 2016). Nevertheless, there is evidence that once infected with *B. xylophilus*, cell death in the plant starts to occur before any major increase in the nematode population, maybe due to the production of phytotoxins (Jones *et al.* 2008). Wilt toxins were found to be produced by nematode-associated bacteria (Oku *et al.* 1980), leading to the hypothesis that bacteria might play an important role in the wilt mechanism (Zhao *et al.* 2014; Nascimento *et al.* 2015; Proença, Grass and Morais 2016).

The microbiome, being the entire collection of microorganisms associated to a given host, carries out functional roles related with host nutrition, pathogen defence and adaptation to environmental changes. Therefore, an organism's health is considered as a result of a network of interactions between microbes and their hosts.

Strategies to control vector-borne diseases are already taking advantage of microbiome functions, including the use of probiotics as plant defence promoters, or disrupting the insect-vector life cycle through manipulation of the host microbiome (Ricci *et al.* 2012; Lebeis 2014; Lacey *et al.* 2015). Following the first suggestions that bacteria may play a role in PWD (Oku *et al.* 1980), various bacterial genera were described in association with *B. xylophilus* (reviewed in Nascimento *et al.* 2015 and Proença, Grass and Morais 2016). Nematode-associated bacteria may promote the wilting process and aid disease development through the production of toxins (as previously mentioned). In addition, a bacterial role in the plant response to the nematode invasion has also been suggested, where bacterial lipases may mediate the production of systemic signalling molecules that trigger plant resistance (Proença *et al.* 2010). Bacteria associated with *B. xylophilus* may also help the nematode tolerate the tree's defence metabolites (i.e. xenobiotic degradation) (Cheng *et al.* 2013; Vicente *et al.* 2013a; Vicente *et al.* 2016).

As previously hypothesized, the microbiome associated with the host tree and the insect vector may also have a determinant role in the disease mechanism (Nascimento *et al.* 2015). In plants and insects, the importance of the associated microbiome is well described. For example, the plant microbiome, besides its role in growth promotion and nutrition improvement, may also activate the plant's immune system (Berg *et al.* 2014; Lebeis 2014). Within vector-borne disease systems, the insect-vector microbiome significantly influences the vector competence (Dennison, Jupatanakul and Dimopoulos 2014). Despite these described microbial functions and their possible relevance in PWD, only a few studies have characterized the bacteria associated with *Monochamus* spp. (Park *et al.* 2007; Vicente *et al.* 2013b; Alves *et al.* 2016) and pine trees (Pirttilä *et al.* 2000; Izumi *et al.* 2008; Proença 2014, Proença *et al.* 2017). With a few exceptions (Vicente *et al.* 2013b; Proença 2014; Alves *et al.* 2016, Proença *et al.* 2017), most of these studies were culture-dependent and thus potentially failed to assess the true structure of the bacterial community (affected by culturing bias). In general, each previous study focused on a single organism (i.e. nematode, insect vector, or tree), and studies used a range of different methods, precluding any definitive conclusion about the bacterial ecology in the context of PWD. Also, to our knowledge, only one metagenomics-based study assessed functional roles of the nematode microbiome (Cheng *et al.* 2013). The analysis of the microbiome, keeping in mind that PWD is a result of interactions among and across multiple distinct organisms, is an essential step towards determining bacterial phylogenetic groups and metabolic pathways that together will diagnose the role of bacteria in the disease mechanism.

The aim of this study was to characterize the microbiomes of *B. xylophilus, P. pinaster*-infected trees and the disease vector *M. galloprovincialis*. Samples of the three organisms were collected during the same time period from the same locations [Comporta and Góis (mainland Portugal) and Madeira Island (Portugal)]. Denaturing gradient gel electrophoresis analysis and pyrosequencing of the 16S rRNA gene were performed to investigate bacterial community structures. The metagenomic content and abundance of gene families were predicted using PICRUSt, focusing on functions previously identified as relevant for disease development.

MATERIALS AND METHODS

Sampling

Sampling was undertaken during May and June 2014 in three different locations where pine forests are known to be affected with PWD (ICNF, 2015): Góis (40°09′07.3″N / 8°07′34.1″W) and Comporta (38°22′48.67″N / 8°47′25.00″W) on the mainland of Portugal; and Prazeres (32°45′45.5″N / 17°11′47.9″W) on Madeira Island (DRFCN 2013; ICNF 2015).

The increase in temperature associated with May and June leads to *B. xylophilus* multiplication in the trees that were likely

infected during late October the previous year (the low temperatures are not favuorable for nematode multiplication) (Mota and Vieira 2008). At the time the samples were collected, the disease was in an advanced stage with visible symptoms, and *M. galloprovincialis* insects were reaching maturation.

In each location, six adult diseased *P. pinaster* trees (˜ 20 years old), with class IV symptoms (Proença *et al.* 2010), were cut. Logs from each tree suspected of having *M. galloprovincialis* pupal chambers were collected. Each log was placed individually in black plastic containers covered with semi-transparent cloth meshes and kept in a greenhouse. A total of 41 insects, caught alive after their emergence from the tree logs, were morphologically identified as *M. galloprovincialis* and sexed. Each insect was surface-sterilized in 70% ethanol solution for 1 min, rinsed in sterile distilled water, and as *B. xylophilus* are primarily located in the vector tracheal system (Kobayashi, Yamane and Ikeda 1984), each insect was dissected under a binocular microscope (Wild Heerbrugg, Switzerland) for trachea extraction. For total DNA and nematode extractions, wood disks (cross-sections) were sawn from the middle and top section of each tree trunk. A total of 36 wood disks were collected, and each was slowly drilled with a sterile spade bit to obtain sawdust from which the DNA was extracted (described later), and the remaining parts of the disk were cut into 1-cm wood pieces for nematode extraction, according to European and Mediterranean Plant Protection Organization (EPPO) guidelines (EPPO 2013). A total of 36 nematode samples were collected and analyzed under a binocular microscope (Wild Heerbrugg, Switzerland) for the morphological confirmation of *B. xylophilus*.

Total genomic DNA extraction

The 36 nematodes samples were washed three times in H_2O_2 (3%) and then washed in distilled water to remove loosely attached microorganisms. Nematode samples were then centrifuged for concentration in a small volume (800 g, 5 min). The 36 samples of sawdust (0.25g from each sample) were re-suspended in 50 μl of sterile Tris-acetate-EDTA (TAE), and insect tracheae were placed in 40 μl of sterile 1X phosphate-buffered saline (PBS). DNA extraction was performed on all samples using PureLink Genomic DNA Mini Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Monochamus species confirmation and detection of *Bursaphelenchus xylophilus*

Insect morphological identification was performed as described in Alves *et al.* (2016). All *P. pinaster*, nematode and *Monochamus* trachea samples were screened for *B. xylophilus* by PCR amplification of the species-specific *MspI* satDNA according to EPPO protocol (EPPO 2013), using the primers and conditions as previously described (Cardoso, Fonseca and Abrantes 2012).

Denaturing Gradient Gel Electrophoresis (DGGE) analysis

A nested PCR technique was applied to amplify the V3 region of the 16S rRNA gene as described in Alves *et al.* (2016). PCR products were run on an 8% polyacrylamide (37.5:1, acrylamide/bisacrylamide) gel with a linear denaturing gradient ranging from 30–65% (100% corresponds to 7 M urea and 40% formamide). Electrophoresis was conducted in a DCode system (Bio-Rad) as described in Henriques *et al.* (2006).

Gel images were acquired using a Molecular Imager® Gel Doc™ XR+ System (Bio Rad Laboratories, Hercules, California, USA). Every DGGE gel contained, at its ends, two lanes with a standard of eight bands for internal and external normalization as an indication of the quality of the analysis (Henriques *et al.* 2006). Replication of the PCR and DGGE steps was performed to confirm the consistency of the profiles.

DGGE patterns were analyzed using Bionumerics software (Applied Maths, Belgium). Cluster analysis of DGGE profiles was performed using the UPGMA (group average) method applying Pearson correlation analysis .

Barcoded 454 pyrosequencing

Considering the DGGE results, a total of 15 samples from different locations were selected for barcoded pyrosequencing (Table 1). 16S rRNA gene amplification was performed as described in Alves *et al.* (2016). The amplicons were quantified by fluorometry with PicoGreen (Invitrogen, CA, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to the manufacturer's instructions (Roche, 454 Life Sciences, USA) at Biocant (Cantanhede, Portugal). Afterwards, each DNA sequence was traced back to its original sample through barcode analysis.

Sequences were processed using both UPARSE (Edgar 2013) and QIIME (Caporaso *et al.* 2010) pipelines as described in Alves *et al.* (2016). Reads were quality-filtered to a maximum expected error of 1.0 and trimmed to 400 bp. Sequences were dereplicated and identical reads were merged, as described in Edgar (2013), generating a file containing a set of unique read sequences each marked with an integer value indicating its abundance. After removal of singletons, Operational Taxonomic Units (OTUs) were defined at 97% similarity by applying the UPARSE-OTU algorithm (which simultaneously identifies and discards chimeras) to the set of unique sequences. Chimera detection was performed a second time using uchime_ref on the generated OTU sequences. Taxonomy assignment was performed within the QIIME environment using Uclust as the assignment method and the Greengenes reference database. At the order level, the Greengenes taxonomic assignment was updated according to the online database classification of List of Prokaryotic names with Standing in Nomenclature (Parte 2013): OTUs assigned to Chitiniphagaceae were included in the Sphingomonadales bacterial order instead of the proposed order Saprospirlales, Actinomycetales was updated to consider the new bacterial orders Micrococcales, Frankiales, Propionobacteriales and Corynebacteriales, and the *Salinispora* genus was included in the phylum Actinobacteria (Table S3).

For alpha-diversity indices, a rarefaction plot of the observed species for each sample was generated using QIIME, along with the richness index, S, Shannon index of diversity, H (Shannon and Weaver 1964), and the equitability index, E (Pielou 1996), calculated using PRIMER v6 software (Anderson, Gorley and Clarke 2008) for each sample as follows:

$$H = -\sum(n_i/N)\ \log\,(n_i/N),$$
$$E = H/\log S,$$

where n_i is the OTU abundance, S is the number of OTUs (used to indicate the number of species) and N is the sum of all reads for a given sample (used as estimates of species abundance) (Fromin *et al.* 2002).

Table 1. Samples selected for the pyrosequencing of *Pinus pinaster* (Pp), *Bursaphelenchus xylophilus* (Bx) and *Monochamus galloprovincialis* (Mg) from the different sampling locations (Comporta—C, Góis—G, Madeira Island—M).

Organism	Location	Samples[a]	Reads	S	J	H
Pinus pinaster	Comporta	Pp.C2	11 561	181	0.690	3.588
		Pp.C4	11 886	135	0.625	3.065
	Góis	Pp.G7	8365	105	0.749	3.484
		Pp.G10	7129	154	0.712	3.586
		Pp.G12	3519	57	0.535	2.161
	Madeira	Pp.M15	2824	204	0.799	4.251
		Pp.M17	2790	202	0.812	4.308
Bursaphelenchus xylophilus	Comporta	Bx.C2	3239	19	0.842	2.479
	Góis	Bx.G10	2144	13	0.477	1.224
		Bx.G12	1897	11	0.542	1.300
	Madeira	Bx.M17	6965	61	0.522	2.146
Monochamus galloprovincialis	Comporta	Mg.C2	2462	16	0.206	0.572
		Mg.C4	9722	17	0.251	0.710
	Góis	Mg.G10	9886	35	0.372	1.323
	Madeira	Mg.M15	3342	18	0.711	2.054
	Total	15	87 731	411		

The number of reads obtained per sample as well as the richness (S), equitability (J) and Shannon (H) indices are shown.
[a]Sample codes were abbreviated for pyrosequencing data. The extended sample codes are in Table S1 in red.

For beta-diversity analysis, the OTU abundance table was firstly rarefied to the lowest number of reads obtained for each sample using QIIME script single_rarefaction.py.

All sequences obtained are available on the NCBI platform with the accession number SRP064549.

Venn diagrams were constructed using the web-based tool package InteractiVenn (Herbele *et al.* 2015).

In silico functional analysis

Functional profiles were explored using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille *et al.* 2013). This bioinformatic tool predicts gene family abundances based on 16S gene surveys using a database of phylogenetically referenced genomes. For the analysis, OTUs were closed-reference selected against the Greengenes database using QIIME according to the online protocol and the functional classification of the predicted genes was performed using KEGG database (Kanehisa *et al.* 2012). The accuracy of metagenome predictions depends on how closely related the microbes in a given sample are to sequenced genome representatives. This accuracy is measured by the Nearest Sequenced Taxon Index (NSTI): lower values indicate a closer relationship (Langille *et al.* 2013).

Statistical analysis

The statistical software R, version 3.1.1 (R Core Team 2014) was used to perform the analyses. PERMANOVA was performed based on 999 permutations to test for significant differences and multi-factor PERMANOVA was used to understand which factors (Species, Location, Tree, and Top/Half height wood, *B. xylophilus* presence) were explaining the variation observed for both the DGGE dataset and for the rarefied OTU abundance table. Both datasets were transformed using the *decostand* function from the *vegan* package in R (Oksanen *et al.* 2015), applying the Hellinger method for transformation; distance matrices were constructed using a Bray-Curtis algorithm.

Indicator orders for each one of the PWD participants were determined using the *multipatt* function from the package *indicspecies* (De Caceres and Legendre 2009) with the species group association parameter (IndVal.g) to correct for unequal group sizes, as described by Fidalgo *et al.* (2016). A significance level of 5% was used for selecting indicators.

Significant differences between indices of richness, diversity and equitability were evaluated using ANOVA when data had a normal distribution (Shaphiro-Wilk tests) and Kruskal-Wallis tests when data did not satisfy the normal distribution assumption of ANOVA.

Cluster analysis of the sequenced samples was performed in PRIMER v6 software using the UPGMA (group average method) on the rarefied and transformed (Hellinger method) OTU table to construct a Bray-Curtis distance matrix.

STAMP software (Parks *et al.* 2014) was used to identify the orthologs with significant differences ($P < 0.05$) in relative abundance between host organisms using ANOVA and Tukey-Kramer *post hoc* tests.

RESULTS

Samples

A total of 18 trees were sampled (six from each sampling location). From these we obtained 36 *P. pinaster* sawdust samples (18 from the middle and 18 from the top section of each tree), 36 nematode and 41 *M. galloprovincialis* samples (Table S1). All *P. pinaster* samples were positive for *B. xylophilus*, confirmed through molecular screening. From the 41 *M. galloprovincialis* samples subjected to *B. xylophilus* molecular detection, 17 were confirmed to contain *B. xylophilus* (12 from Comporta, three from Góis and two from Madeira Island).

All nematode samples were positive for *B. xylophilus* through morphological and molecular analysis (data not shown). However, only eight samples were positive in the first amplification of the two-step PCR assay, suggesting low numbers of *B. xylophilus* in the remaining 28 samples. These were excluded from further analysis (Table S1).

DGGE analysis

DGGE analysis was used to compare the bacterial community structures among samples. A total of 85 samples were selected for DGGE analysis: eight nematodes, 36 *P. pinaster* and 41 *M. galloprovincialis* (Table S1). DGGE profiles of within-sample technical replicates showed high reproducibility between gels and PCR amplifications (data not shown). Cluster analysis of DGGE profiles showed three main clusters corresponding to the majority of the *P. pinaster* , *B. xylophilus* and insect samples (Fig. S1). Multi-factor PERMANOVA analysis of the DGGE dataset (Table S2) showed that the factor which explained most of the variation was the organism species ($R^2 = 0.287$, $P < 0.05$), with significant differences among the bacterial community profiles across all three species. Sampling location also had a significant impact ($P < 0.05$, PERMANOVA) in *P. pinaster* and *M. galloprovincialis* bacterial communities. Given the insufficient number of nematode samples, it was not possible to determine the impact of the sampling location in the bacterial community of *B. xylophilus*. From each tree, sawdust samples collected from the top and middle sections of the trunk were compared by DGGE and no significant differences were observed (PERMANOVA, $P > 0.05$). In addition, there were no significant differences between insects with or without *B. xylophilus* (PERMANOVA, $P > 0.05$).

Pyrosequencing results

Fifteen samples were selected for pyrosequencing, seven from *P. pinaster*, four from *B. xylophilus* and four from *M. galloprovincialis* carrying *B. xylophilus* (Table 1). Samples were selected to represent the main clusters identified through DGGE analysis. Considering the differences among sampling areas suggested by DGGE analysis, samples from each area were included in the sequencing analysis to obtain a more realistic picture of the microbiome of each organism.

A total of 93112 reads were obtained, which clustered in 414 OTUs after quality-filtering, chimera-checking and the removal of singletons. Three OTUs corresponded to chloroplast DNA and were discarded from the analysis. The final dataset consisted of a total of 87731 high-quality reads, corresponding to 411 OTUs (Table 1). The rarefaction curve for each sample reached saturation (Fig. S2), indicating that the OTUs detected were representative of the bacterial communities.

From the 411 OTUs, nine were not assigned to any phylum (corresponding to 0.1% of the total number of reads) (Table S3).

Bacterial community structures associated with *B. xylophilus*, *M. galloprovincialis* and *P. pinaster*

A total of 393 OTUs were detected in *P. pinaster* samples that assigned to 11 different bacterial phyla (Table S4). The most abundant phylum was Proteobacteria with an average of 68.2% (± 10.9% SD) of the total number of reads for each tree's samples, followed by Acidobacteria (19.9 ± 7.9%). *P. pinaster* OTUs were assigned to 42 different bacterial orders. Xanthomonadales, Acidobacteriales and Rhizobiales were the most abundant orders, altogether accounting for 59.0% of the total number of *P. pinaster* reads (Fig. 1). For *P. pinaster* there were 14 indicator orders (Table S6), of which six were represented by > 1% of the total reads: Xanthomonadales, Acidobacteriales, Rhizobiales, Sphingobacteriales, Micrococcales and Sphingomonadales. The most represented OTUs were OTU 3 (9.3% of *P. pinaster* total reads) and OTU 4 (8.1% of *P. pinaster* total reads) that assigned with Xanthomonadaceae and Acidobacteriaceae, respectively. These two OTUs belong to the group of 12 OTUs that were present in all *P. pinaster* samples and that represented 26.4% of the total number of reads. These OTUs were assigned to Xanthomonadaceae, Acidobacteriaceae Rhizobiaceae, Caulobacteraceae and Sphingomonadaceae.

A total of 76 OTUs were detected in *B. xylophilus* samples that were assigned to seven different phyla with Proteobacteria being the most abundant (93.0 ± 7.3%). Among the 12 different bacterial orders detected, Pseudomonadales and Enterobacteriales were dominant, representing 54.2% of the nematode sample reads. The exception was the nematode sample from Madeira (Bx.M17), in which the most abundant order was Xanthomonadales (accounting for 46% of the reads in this sample). No indicator orders were detected for nematode samples. The most abundant OTUs were OTU 3 (Xanthomonadaceae) and OTU 2 (Enterobacteriaceae), represented by ˜18% of the *B. xylophilus* total reads. There were three OTUs shared between all samples (OTU 27, 82, 264) belonging to Pseudomonadaceae and Enterobacteriaceae families, representing 14.5% of the total number of reads (Fig. 2).

The 49 OTUs detected in *M. galloprovincialis* samples assigned to five different phyla, with Proteobacteria being the most abundant (97.1 ± 3.6%). The most abundant order in all samples was Pseudomonadales (74.4 ± 9.1%). There were three indicator orders for *M. galloprovincialis* with Pseudomonadales and Rhodobacterales being the most abundant (> 1% of the reads). At the OTU level, the most represented were OTU 1 (62.2% of *M. galloprovincialis* total reads) and OTU 2 (9.4% of *M. galloprovincialis* total reads), that assigned to Pseudomonadaceae and Enterobacteriaceae, respectively.

The cluster-based analysis of bacterial community structure and composition (as OTUs) showed two main clusters and indicated that the nematode samples generally grouped with the samples from the insect vector (Fig. 1). PERMANOVA analysis of the pyrosequencing data showed that the *P. pinaster* bacterial community was significantly different from both the insect and nematode bacterial communities ($P < 0.05$). There were no significant differences between the insect and nematode bacterial communities ($P > 0.05$). In terms of alpha-diversity, significant differences in diversity and richness indices were observed between *P. pinaster* (higher values) and the other two species ($P < 0.05$, one-way ANOVA) (Table 1). Despite these differences, 22 OTUs were shared between *M. galloprovincialis*, *B. xylophilus* and *P. pinaster*, corresponding to 47.8% of the total number of reads (Figs 2 and 3). These OTUs were assigned to eight different bacterial families, among which Enterobacteriaceae (five OTUs), Pseudomonadaceae (four OTUs) and Burkholderiaceae (four OTUs) included the highest number of shared OTUs (Fig. 3). There were only three OTUs shared by the three species analyzed from the three sampling locations: OTU 19 (Enterobacteriaceae, represented by 1.3% of the total reads), OTU 82 and OTU 27 (both from Pseudomonadaceae, 3.3%). Insect samples shared 44.9% of their OTUs with *B. xylophilus*, while *P. pinaster* shared 17.6% with the nematode (Fig. 2a).

Influence of location on PWD bacterial community

The number of sequenced samples was insufficient to draw statistically robust conclusions regarding the influence of location on PWD microbiome. Nevertheless, it is worth mentioning that the pyrosequencing results indicate differences in bacterial composition of samples from different geographical areas,

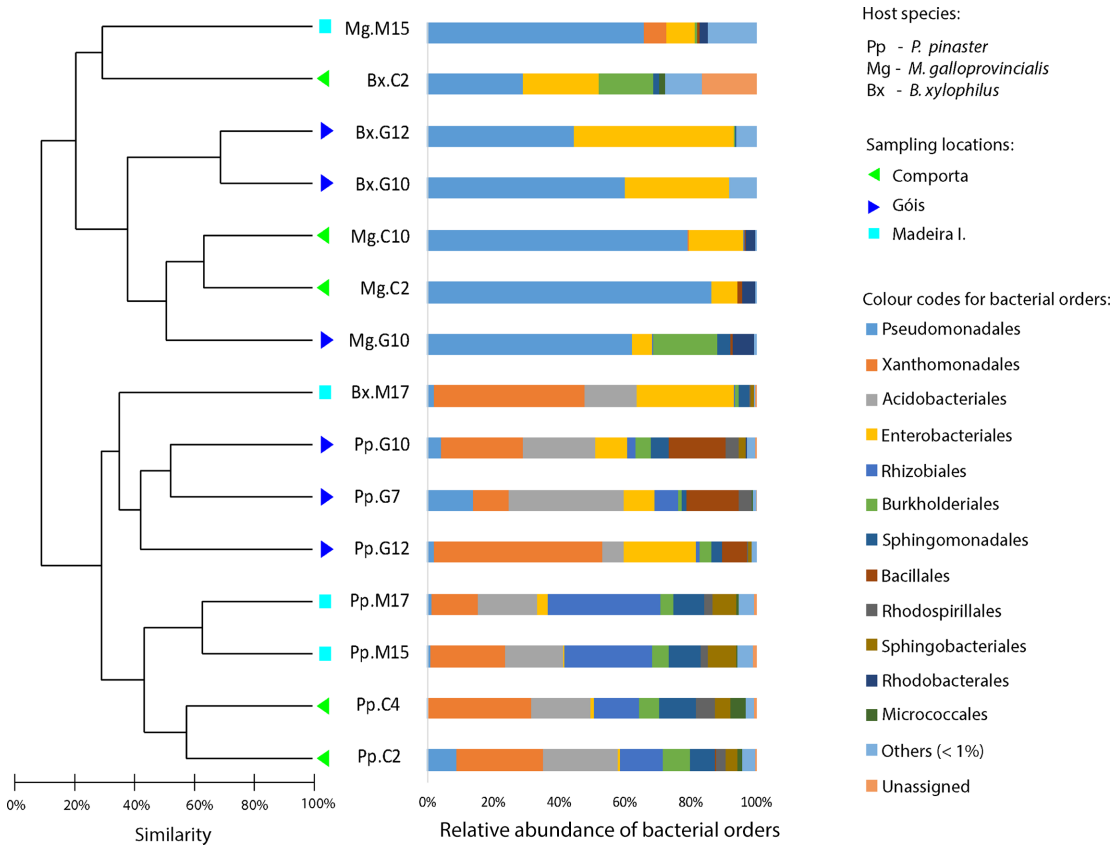

Figure 1. Bacterial community structure and composition analysis for the sequenced samples. The dendrogram of the bacterial community structure was created using the UPGMA method. Relative abundance of the predominant bacterial orders is plotted for each sample.

in agreement with the findings resolved through DGGE analysis. For *P. pinaster*, Rhizobiales (29.2 ± 4.1%) was the most abundant bacterial order on Madeira Island, but this was not consistent with samples from the mainland. In nematodes from Madeira Island, Xanthomonadales was the most abundant bacterial order. In addition, Acidobacteriales was only detected in Madeira Island nematodes and not in mainland samples (Fig. 1). For *M. galloprovincialis*, Lactobacillales were detected only in Madeira Island samples.

At the OTU level, there were different groups of shared OTUs in samples collected from each of the three locations (Figs 2b, c and d): mainland organisms shared the presence of OTU 1 (Pseudomonadaceae, 16681 reads, 23.2% of total mainland reads), which was not detected in samples collected on Madeira Island; Comporta samples shared OTU 197 (Enterobacteriaceae, 87 reads, 0.22% of total Comporta reads); Góis samples shared eight OTUs (5730 reads) that were not present in the samples from other locations, belonging to five different families and including 17.4% of the total Góis reads (Fig. 3). Altogether, these results support the apparent site-specific differences in PWD-associated microbiomes as revealed by the DGGE profiles.

In silico metagenome analysis

Metagenomic inference from the pyrosequencing results was applied and the accuracy of the inferences assessed through NSTI values. The mean NSTI values were 0.068 ± 0.015 for *P. pinaster* samples, 0.052 ± 0.014 for *M. galloprovincialis* samples, and 0.038 ± 0.015 for *B. xylophilus* samples, indicating the high accuracy of the PICRUSt predictions.

A total of 5618 functional orthologs were predicted to be present in the analyzed bacterial communities (Table S7), corresponding to 41 Level 2 KEGG pathways. For *P. pinaster* samples an average of 4985 functional orthologs were predicted with a standard deviation of 157, 4509 ± 223 for *M. galloprovincialis* and 3980 ± 325 for *B. xylophilus*. The most abundant Level 2 KEGG pathways (> 3% of relative abundance) are shown in Fig. 4, comprising functions including membrane transport systems, and the metabolism of amino acids and carbohydrates.

For each microbiome, KEGG orthologs (KOs) corresponding to functions previously described as relevant for the host and/or for PWD development were analyzed. Specifically, the bacterial metagenomes of the insect and *B. xylophilus* were analyzed for the presence of KOs related with xenobiotic degradation and phytopathogenicity to evaluate the potential of both microbiomes to aid the nematode in weakening the tree and surviving defensive compounds. Xenobiotic degradation pathways were considered relevant for the predicted functional metagenomes once they reached > 3% of relative abundance (Fig. 4; 3.745 ± 0.470% for *M. galloprovincialis* and 3.475 ± 0.431% for *B. xylophilus*). A complete 3-oxoadipate pathway for benzoate degradation (Fig. S3, adapted from KEGG pathway map00362, Kanehisa *et al.* 2012) was reconstructed both for the insect and nematode based on the deduced metagenome. Other KOs related to xenobiotic degradation were predicted at relevant abundances such as enoyl-CoA-hydratase, involved in limonene and alpha-pinene degradation (0.148 ± 0.036% for *M. galloprovincialis*, 0.117 ± 0.051% for *B. xylophilus*), and p-hydroxybenzoate 3-monooxygenase and 4-carboxymuconolactone decarboxylase, which are involved in the degradation of aromatic compounds. Related

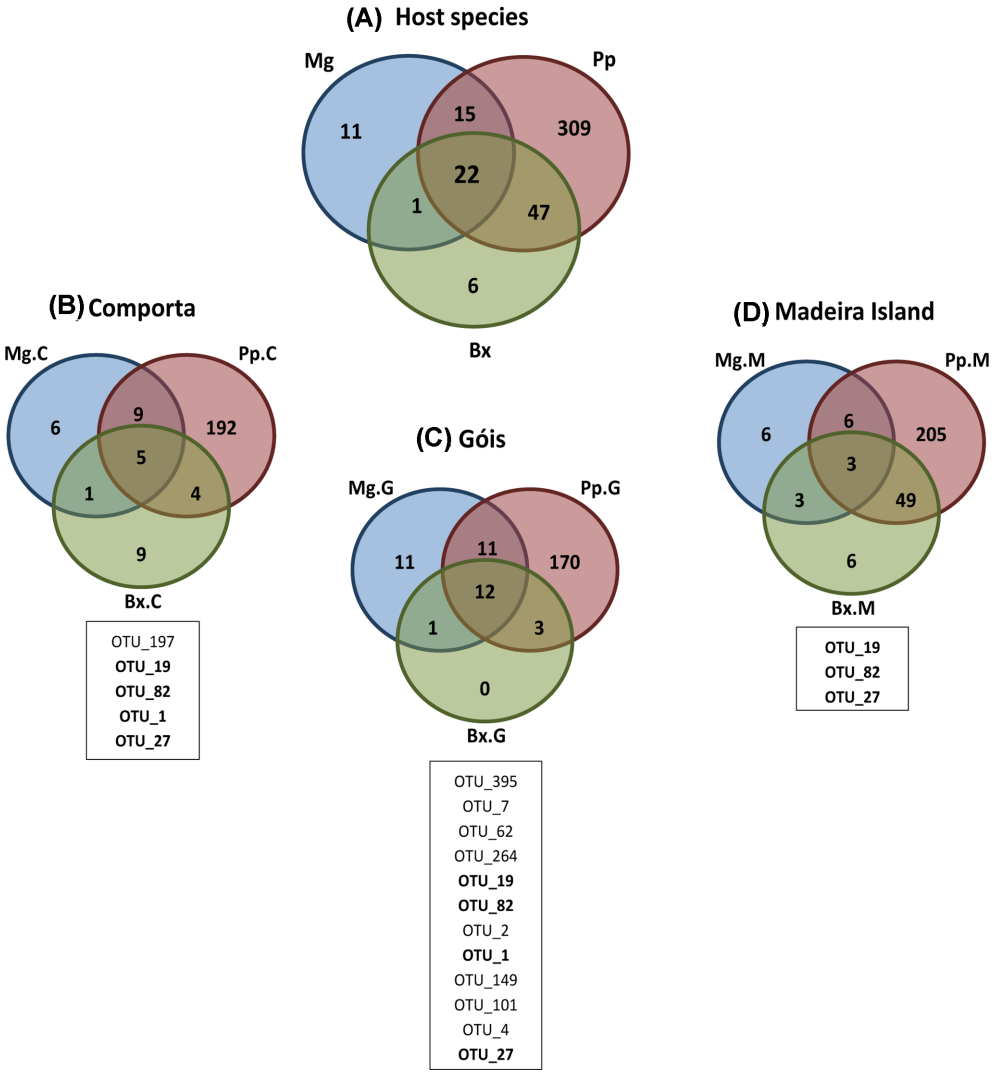

Figure 2. PWD core microbiome. Venn diagrams representing (A) the shared OTUs between *Monochamus galloprovincialis* (Mg), *Pinus pinaster* (Pp) and nematodes (Bx); and the shared OTUs between the samples collected in (B) Comporta, (C) Góis and (D) Madeira Island. The shared OTUs for each location are listed below the corresponding Venn diagram.

to the oxidative stress response, orthologs for catalase, peroxidase and superoxide dismutase were predicted for all samples, both from the insect and nematode. Catalase KOs had the highest abundances across all samples (0.038 ± 0.002% for *M. galloprovincialis* and 0.041 ± 0.010% for *B. xylophilus*).

Regarding bacterial phytopathogenicity, several orthologs were inferred for all samples of both organisms: orthologs related with type III secretion systems and the repeat-in-toxin (RTX) transporter KO; orthologs that would facilitate the phytopathogens' host cell invasion (e.g. adhesion/invasion and endoglucanase, endo-1,4-beta-xylanase and pectinesterase KOs); orthologs for proteases; and for the exopolysaccharide PGA (poly-beta-1,6-N-acetyl-D-glucosamine; *pgaABCD)*. Regarding the production of phytotoxins or other potential plant-damaging compounds, the complete metabolic pathway for phenilacetic acid synthesis was predicted in the nematode microbiome as showed in Fig.5 (adapted from KEGG pathway map00360, Kanehisa *et al.* 2012), and pyochelin synthetase was predicted in some of the insect and nematode samples. Both compounds were previously described as potential pathogenic factors in PWD development and were produced by PWN-associated bacteria (Kawazu *et al.* 1996; Le Dang *et al.* 2011).

For the tree microbiome, KEGG orthologs related to different plant growth promotion traits were predicted in all *P. pinaster* samples: KOs for indolepyruvate decarboxylase, isochorismate pyruvate-lyase, and 1-aminocyclopropane-1-carboxylate deaminase, which are implicated in the syntheses of the plant hormones indol-3-acetic acid, salicylic acid and ethylene, respectively; orthologs for *nifDKH* genes which are necessary for nitrogen fixation and phytases for phosphate solubilization; and chitinases that might be involved in plant defence. Orthologs for oxidative stress-related enzymes, including catalases, glutathione-S-transferase, and glutathione-S-peroxidase, were also predicted, and were abundant in all *P. pinaster* samples (0.033 ± 0.005%, 0.015 ± 0.007%, 0.039 ±0.007%, respectively). On the other hand, phytopathogenicity-related genes (e.g. for bacterial motility, adhesion/invasion and host cell wall degradation) were also predicted in all tree samples. Among these are orthologs for the flagellar synthesis of proteins, endoglucanase, polyglacturonase, pectinesterase and endo-4,5-beta xylanase,

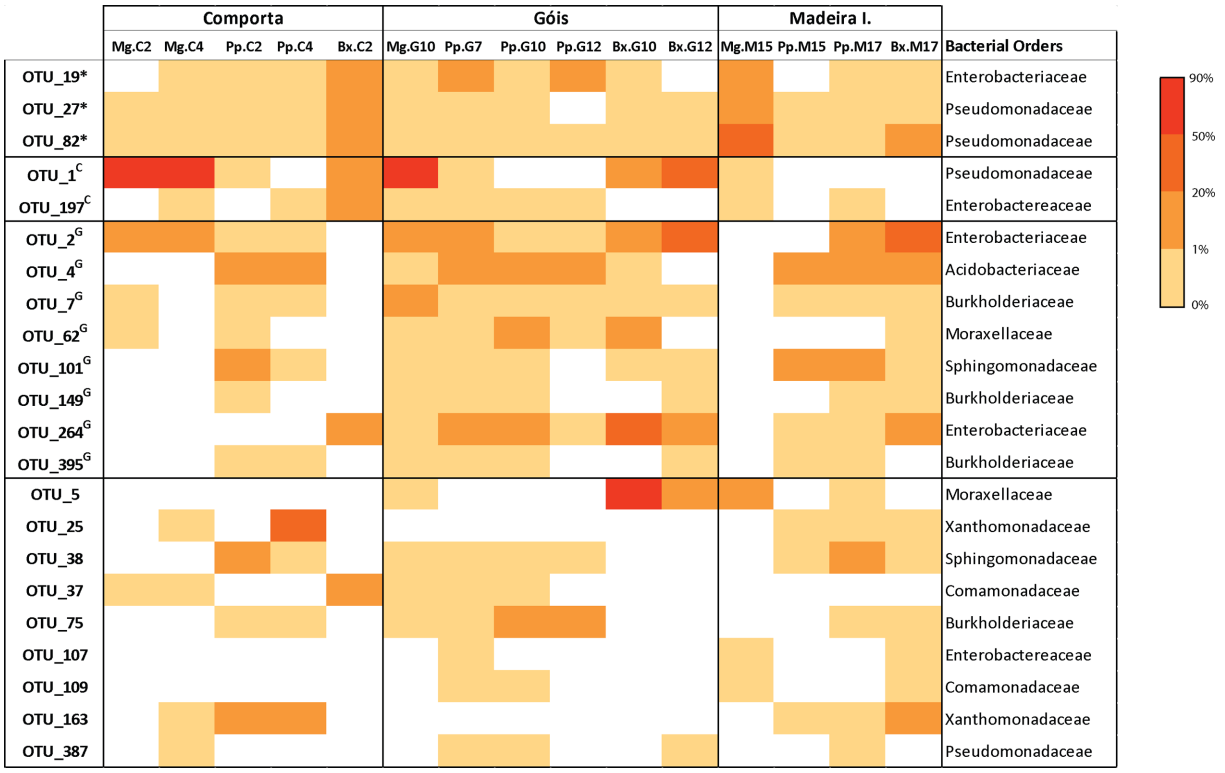

Figure 3. Heatmap for the 22 PWD core OTUs. The colour codes represent the relative abundance of OTUs in each sample. The PWD OTUs present in all sampling locations are indicated by ∗, C (only in Comporta) and G (only in Góis).

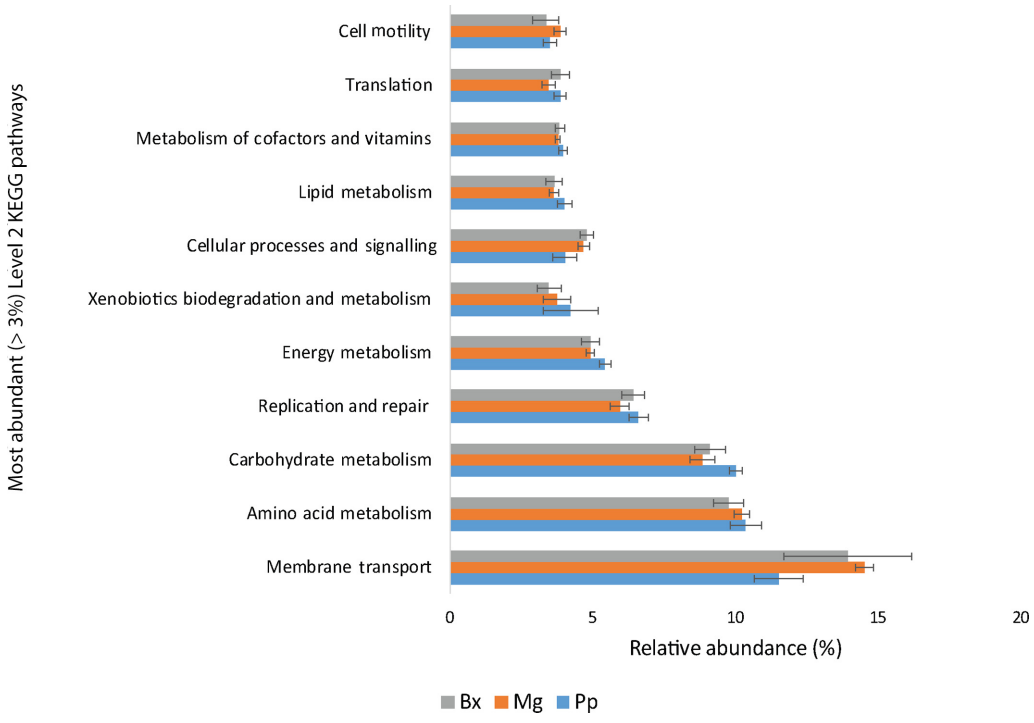

Figure 4. Relative abundance of the most represented (> 3%) Level 2 KEGG pathways predicted for the three PWD organisms.

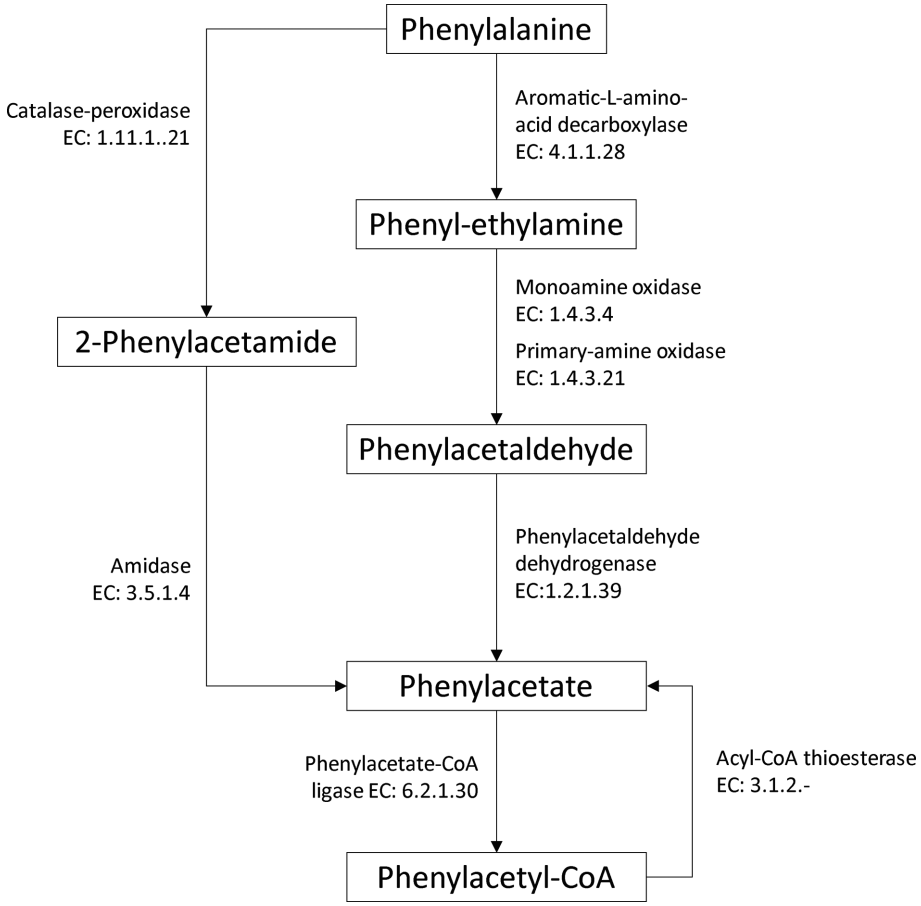

Figure 5. Enzymes predicted to be present in *B. xylophilus* microbiome for phenilacetate synthesis. Based on the KEGG pathways map for phenylalanine metabolism (map00360; http://www.genome.jp/kegg-bin/show_pathway?map00360).

opines' transport systems, and MFS (major facilitator superfamily) transporters. Type III (0.012 ± 0.007%) and type IV secretion systems were predicted in all samples, with higher relative abundances of type IV secretion system-related KOs (0.214 ± 0.102%).

DISCUSSION

This is the first study to assess the bacterial community associated with PWN, the insect vector, and infected *P. pinaster* trees, using samples collected simultaneously from the same affected sites. DGGE allowed the comparison of a high number of samples concluding that, as expected, the microbiome structure relates mainly to the identity of the host's species. However, sampling location was also identified as a significant factor driving differences in the *P. pinaster and M. galloprovincialis* microbiomes. The DGGE results reinforce the importance of spatial factors when assessing the microbiome of these organisms. Through the utilization of pyrosequencing, this study provided a description of the infected *P. pinaster* trees' microbiome, which has been only rarely and partially assessed.

This bacterial community was dominated by Xanthomonadales, Acidobacteriales and Rhizobiales, which were among the 14 orders flagged as indicator bacterial orders for this microbiome. Xanthomonadales, Acidobacteriales and Rhizobiales have previously been described in association with *P. pinaster*, including within infected trees (Proença 2014; Proença *et al.* 2017). The most abundant OTUs present in all *P. pinaster* samples (OTU 3 and 4) were taxonomically assigned to Xanthomonadeceae and Acidobacteriaceae bacterial families. In self-defence, plants intensify the production of a cocktail of aromatic and xenobiotic compounds (Huot, Nachappa and Tamborindeguy 2013). As described by Futai (2013), an accumulation of polyphenols occurs during the disease development. In addition, as described for *Pinus sylvestris*, during wood decay a significant drop in pH occurs (Kielak *et al.* 2016). Altogether, these conditions in the infected trees may exert a selective pressure on the plant microbiome, modulating the bacterial community structure and selecting for resistant microorganisms. For instance, bacteria belonging to the Xanthomonadaceae bacterial family (*Stenotrophomonas* spp.) were reported as being able to degrade aromatic compounds and other xenobiotics (Mangwani *et al.* 2014; Tiwari *et al.* 2016), and Acidobacteriaceae is a bacterial group with optimal growth at low pH (Rawat *et al.* 2012). Therefore, the persistence of both bacterial groups may be favoured in the infected *P. pinaster* trees. Furthermore, members of the bacterial order Rhizobiales, detected as abundant in this study, are known as plant growth-promoting bacteria mainly due to their ability for nitrogen fixation (Hardoim *et al.* 2015), which is useful for their host.

Despite the indubitable beneficial role of plant-associated bacteria, it is also possible that under specific conditions the microbiome may facilitate disease establishment. For instance, in the olive knot disease, bacterial endophytes cooperate with the bacterial pathogen and modulate disease severity (Buonaurio *et al.* 2015). Furthermore, tomato-associated bacteria can aid

the infection of the rot-knot nematode, *Meloidogyne incognita* (Tian *et al.* 2015). In PWD the changes in the tree physiology due to disease progression may lead to changes in the bacterial community functions, and the endophytes that were once beneficial may turn harmful. For instance, the Xanthomonadaceae family has been reported in association with both healthy and diseased plants, and these family members could be both endophytic and phytopathogenic. Indeed, there are several bacterial species belonging to the Xanthomonadaceae family included in the A1 quarantine pest list of the European and Mediterranean Plant Protection Organization (EPPO, 2014).

The *in silico* predicted metagenomic functions of the infected tree microbiome also support the aspects discussed above. On one hand, several orthologs implicated in the syntheses of plant hormones and nitrogen fixation were detected; however, type III and type IV secretion systems were also predicted in all tree samples. These traits are related with plant growth promotion and phytopathogenicity, respectively (Hardoim *et al.* 2015). In summary, the tree microbiome was shown to possess the potential to assist the tree defence, but may also switch roles as the disease develops, ultimately contributing to tree decay. These results highlight the need to better understand the temporal dynamics of the tree-associated microbiome during the progression of different PWD stages.

Besides the importance for the nematode life cycle, bacteria may play a role helping the nematode development inside the tree. Tree resin compounds such as phenolic compounds and polycyclic aromatic hydrocarbons are harmful for the nematode; however, these can potentially be degraded/detoxified to benzoate and then enter the benzoate degradation pathway. As previously described (Cheng *et al.* 2013), complete pathways for the degradation of benzoate were predicted in the nematode microbiome. Additionally, members of Enterobacteriales found in association with *B. xylophilus* were described as producers of cell wall-degrading enzymes (Vicente *et al.* 2012b) that would possibly assist the nematode invasion inside the tree. This is supported by the fact that orthologs related with endoglucanases, endo-1,4-beta-xylanases and pectinesterases, were predicted for all nematode samples. As expected, orthologs for proteases and serine proteases were also predicted, in accordance with previous studies based on cultivation of nematode-associated bacteria (Proença *et al.* 2010). Our results support the hypothesis that the nematode microbiome might assist its host in cell wall degradation, tissue penetration, and digestion of tree proteins. As previously suggested (Vicente *et al.* 2013b), the nematode microbiome may also contribute to neutralize H_2O_2 by producing catalases and peroxidases (predicted in the nematode microbiome), possibly helping the nematode to cope with oxidative stress inside the tree. It has been suggested that once infected with *B. xylophilus*, cell death in the plant starts to occur before any major increase in the nematode population due to the production of phytotoxins (Oku *et al.* 1980; Jones *et al.* 2008). In the nematode-deduced metagenome, we found the complete pathway for phenilacetic acid production and also orthologs in some samples for pyochelin, both described as compounds potentially involved in the tree-wilting process (Kawazu *et al.* 1996; Le Dang *et al.* 2011).

In the *M. galloprovincialis* samples, the most abundant indicator orders were assigned to Pseudomonadales and Rhodobacterales. Species from these groups were described as being able to degrade toxic compounds (Bartosik, Szymanik and Baj 2003; Cheng *et al.* 2013). The functional predictions for the insect bacterial community revealed high adaptation to the plant-hostile environment, for example, through chemical detoxification pathways. The orthologs detected for the insect samples predict a complete 3-oxidopate pathway for benzoate degradation, as described for the nematode microbiome. Besides the promotion of host fitness and survival, the bacteria associated with *Monochamus* may help to maintain the nematode in the tracheae allowing for transportation and transmission: bacteria may sequester the nutrients and low-nutrient concentration maintains the nematode in its resistance stage, as described for the malaria mosquito vectors (Dennison, Jupatanakul and Dimopoulos 2014). A question raised in previous studies is whether the nematode carries a species-specific microbiome. No indicator orders were predicted for the nematode samples. Low values of richness and diversity were determined for the *B. xylophilus* microbiome, which is in agreement with previous studies with entomophilic nematodes (Koneru *et al.* 2016). In this study nematodes and insects shared a substantial percentage of their OTUs with no significant differences between both microbiomes. On the other hand, results reveal that during its stage inside the tree the nematode has its own bacterial community with a significantly different composition from the tree microbiome. Thus, our results support the hypothesis that the nematode microbiome may, at least in part, be inherited from its insect vector, and is maintained during the stage when it transitions to life inside the tree, suggesting an importance to the nematode's survival.

As stated above, DGGE analysis revealed significant differences in terms of the microbiome structure among sampling locations. Despite the low number of samples, these differences were also reflected in the pyrosequencing data, altogether suggesting that each location had different dominant bacterial groups. Specifically, samples from Madeira Island had higher richness and diversity.

The changes in the microbiome of each organism may provide physiological adaptations to the environmental conditions, as described for cryptic species of nematodes (Derycke *et al.* 2016). There are some dissimilarities in climate across the site locations: Calheta, in Madeira Island, is at an altitude of 121 m and has a Csb (warm summer Mediterranean) climate according to the Kppen and Geigen classification (Peel, Finlayson and McMahon 2007), with an average temperature of 18.1°Cand average precipitation of 593 mm. On the mainland, Comporta and Góis also have Csa climates, but the former is located only 2 m above sea level, with an average temperature of 17.2°Cand average precipitation of 958 mm. Góis is at an altitude of 195 m with an average temperature of 15.7°C and a mean precipitation of 958 mm. The results obtained suggest the existence of environment-specific bacteria, probably a reflection of the adaptation to the environment specificities as described by Lebeis (2014). Studies from the Madeira Island Regional Government (Município do Funchal 2016) indicate that the Madeira Island climate, with mild annual temperatures, influence the insect-vector life cycle where insects are capable of flight all year. Consequently, the disease dispersion potential is affected, with higher rates reported for the island than on the mainland. These differences in the disease cycle between locations are not thought to be the result of molecular differences in *B. xylophilus*, which have been proven to be similar across the mainland and Madeira Island (Fonseca *et al.* 2012). Yet disease cycle variations may have consequences in the microbial ecology of the disease and may lead to the observed differences in this study between locations in the bacterial structure. Previous studies have described the influence of tree geographical origin

in nematode infection, showing differences related to pine physiology, nematode migration ability, speed of the wilt symptom development, and death of the infected trees (Zas *et al.* 2014; Hopf-Biziks, Schröder and Schütz 2016). Future studies, with increased sampling, should explore the effect of spatial factors on the microbiome of PWD and on the development of the disease.

CONCLUSIONS

This study presents an in-depth characterization of the microbiome associated with PWN, the insect vector and infected *P. pinaster* trees, which has proven essential in understanding the role of this community in the disease development and to identify targets for biological control.

Direct effects of the nematode and insect microbiomes in PWD (e.g. toxin production, degradation of xenobiotics) seem plausible and are supported by the results obtained from metagenomic functional inference. The microbiome associated to the tree can exert both positive and negative effects on the tree, depending on the disease stage. Thus, the disease must be understood as a synergy between different organisms with much to be discovered about the interactions between these organisms and the microbiome.

Considering PWD control, both shared and specific bacterial groups identified in this study might be potential targets to suppress important processes, leading to interruption of the disease cycle. Further analysis of the data collected during this study on the PWN, the insect vector and the infected *P. pinaster* trees' microbiome may also help identify bacterial groups with the potential to be used as probiotics, for example to strengthen tree defences.

The comparison of the microbiomes from three affected sampling locations revealed some indication of changes in the bacterial composition between sampling locations. The adjustments of the microbiome across the biogeographical range of the disease may impact the PWD mechanism, and should be considered in the establishment of bacterial targets for disease control.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of interest. None declared.

REFERENCES

Abelleria A, Picoaga A, Mansilla JP *et al.* Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in North — western Spain. *Am Phytopathol Soc* 2011;**95**:776.

Alves M, Pereira A, Matos P *et al.* Bacterial community associated to pine wilt disease insect vectors *Monochamus galloprovincialis* and *Monochamus alternatus*. *Sci Rep* 2016, **6**, 23908.

Anderson MJ, Gorley RN, Clarke KR. *PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods*. UK: Plymouth, 2008.

Bartosik D, Szymanik M, Baj J. Identification and distribution of insertion sequences of *Paracoccus solventivorans*. *Appl Environ Microbiol* 2003;**69**:7002–7008.

Berg G, Grube M, Schloter M *et al.* The plant microbiome and its importance for plant and human health. *Front Microbiol* 2014;**5**:148.

Buonaurio R, Moretti C, Passos da Silva D *et al.* The olive knot disease as a model to study the role of interspecies bacterial communities in plant disease. *Front Plant Sci* 2015;**6**:434.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;**7**:335–336.

Cardoso JMS, Anjo SI, Fonseca L *et al.* *Bursaphelenchus xylophilus* and *B . mucronatus* secretomes: a comparative proteomic analysis. *Sci Rep* 2016;**6**:39007.

Cardoso JMS, Fonseca L, Abrantes I. Direct molecular detection of the pinewood nematode, *Bursaphelenchus xylophilus*, from pine wood, bark and insect vector. *Eur J Plant Pathol* 2012;**133**:419–425.

Cheng X-Y, Tian X-L, Wang Y-S *et al.* Metagenomic analysis of the pinewood nematode microbiome reveals a symbiotic relationship critical for xenobiotics degradation. *Sci Rep* 2013;**3**:1869.

De Carceres M, Legendre P Associations between species and groups of sites: indices and statistical inference. *Ecology* 2009;**90**:3566–74.

Dennison NJ, Jupatanakul N, Dimopoulos G. The mosquito microbiota influences vector competence for human pathogens. *Curr Opin Insect Sci* 2014;**3**:6–13.

Derycke S, De Meester N, Rigaux A *et al.* Coexisting cryptic species of the *Litoditis marina* complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability. *Mol Ecol* 2016;**25**:2093–2110.

Direcção Regional de Floresta e Conservação da Natureza (DRFCN). *Plano de acção para controlo do nométodo da madeira do pinhiero (NMP) na região autónoma da madeira (RAM)*, 2013.

Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013;**10**:996–998.

Espada M, Silva AC, Eves Van Den Akker S *et al.* Identification and characterization of parasitism genes from the pinewood nematode *Bursaphelenchus xylophilus* reveals a multilayered detoxification strategy. *Mol Plant Pathol* 2015;**17**:286–295.

European and Mediterranean Plant Protection Organization (EPPO). EPPO A1 and A2 lists of pests recommended for regulation as quarantine pests. *EPPO Standard PM1/2 (23)* 2014.

European and Mediterranean Plant Protection Organization (EPPO). PM 7/4 (3) *Bursaphelenchus xylophilus*. *EPPO Bulletin* 2013;**4**:105–118.

Fidalgo C, Henriques I, Rocha J *et al*. Culturable endophytic bacteria from the salt marsh plant *Halimione portulacoides*: phylogenetic diversity, functional characterization, and influence of metal(loid) contamination. *Environ Sci Pollut R* 2016;**23**:10200–10214.

Fonseca L, Cardoso JMS, Lopes A *et al*. The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. *Helminthologia* 2012;**49**:96–103.

Fromin N, Hamelin J, Tarnawski S *et al*. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ Microbiol* 2002;**4**:634–643.

Futai K. Pine Wood Nematode. *Annu Rev Phytopathol* 2013;**51**:61–83.

Hardoim PR, van Overbeek LS, Berg G *et al*. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol Mol Biol Rev* 2015;**79**:293–20.

Henriques IS, Alves A, Tacão M *et al*. Seasonal and spatial variability of free-living bacterial community composition along an estuarine gradient (Ria de Aveiro, Portugal). *Estuar, Coast Shelf S* 2006;**68**:139–148.

Herbele H, Meirelles GV, da Silva FR *et al*. InteractiVenn: a web-based tool for analysis of sets through Venn diagrams. *BCM Bioinformatics* 2015;**16**:169.

Hopf-Biziks A, Schröder T, Schütz S. The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, and its pathogenicity to German *Pinus sylvestris* provenances. *J Plant Dis Protect* 2016;**123**:43–49.

Huot OB, Nachappa P, Tamborindeguy C The evolutionary strategies of plant defenses have a dynamic impact on the adaptations and interactions of vectors and pathogens. *Insect Sci* 2013;**20**:297–306.

Instituto da Conservação da Natureza e das Florestas (ICNF). *Nemátodo-da-madeira-do-pinheiro/Informação geográfica*, 2015.

Inácio ML, Nóbrega F, Mota M *et al*. First detection of Bursaphelenchus xylophilus associated with Pinus nigra in Portugal and in Europe. *For Pathol* 2014;**45**:235–38.

Izumi H, Anderson IC, Killham K *et al*. Diversity of predominant endophytic bacteria in European deciduous and coniferous trees. *Can J Microbiol* 2008;**54**:173–179.

Jones JT, Moens M, Mota M *et al*. *Bursaphelenchus xylophilus*: opportunities in comparative genomics and molecular host-parasite interactions. *Mol Plant Pathol* 2008;**9**:357–368.

Kanehisa M, Goto S, Sato Y, *et al*.. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res* 2012;**40**:D109–114.

Kawazu K, Zhang H, Yamashita H *et al*. Relationship between the pathogenicity of the pine wood nematode, *Bursaphelenchus xylophilus* and phenylacetic acid production. *Biosci, Biotech, and Bioch* 1996;**60**:1413–1415.

Kielak AM, Scheublin TR, Mendes LW *et al*. Bacterial community succession in pine-wood decomposition. 2016;7:231. *Front Microbiol* 2016;**7**:231.

Kikuchi T, Cotton JA, Dalzell JJ *et al*. Genomic insights into the origin of parasitism in the emerging plant pathogen *Bursaphelenchus xylophilus*. *PLoS Pathog* 2011;**7**:e1002219.

Kobayashi F, Yamane A, Ikeda T. The japanese pine sawyer beetle as the vector of pine wilt disease. *Annu Rev Entomol* 1984;**29**:115–135.

Koneru SL, Salinas H, Flores GE *et al*. The bacterial community of entomophilic nematodes and host beetles. *Mol Ecol* 2016;**25**:2312–2324.

Lacey LA, Grzywacz D, Shapiro-Ilan DI *et al*. Insect pathogens as biological control agents: back to the future. *J Invertebr Pathol* 2015;**132**:1–41.

Langille MGI, Zaneveld J, Caporaso JG *et al*. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 2013;**31**:814–821.

Lebeis SL. The potential for give and take in plant-microbiome relationships. *Front Plant Sci* 2014;**5**:287.

Le Dang Q, Son SW, Cheon H *et al*. Pyochelin isolated from *Burkholderia arboris* KRICT1 carried by pine wood nematodes exhibits phytotoxicity in pine callus. *Nematology* 2011;**13**:521–528.

Mangwani N, Shukla SK, Kumari S *et al*. Characterization of *Stenotrophomonas acidaminiphila* NCW-702 biofilm for implication in the degradation of polycyclic aromatic hydrocarbons. *J Appl Microbiol* 2014;**117**:1012–24.

Mota M, Vieira P *Pine Wild Disease: a worldwide threat to forest ecosystems*. Springer, 2008.

Mota MM, Braasch H, Bravo MA *et al*. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1999;**1**:727–734.

Município do Funchal *Espécie do mês/Nemátode-da-Madeira-do-Pinheiro*, 2016.

Nascimento FX, Hasegawa K, Mota M *et al*. Bacterial role in pine wilt disease development -review and future perspectives. *Env microbiol rep* 2015;**7**:51–63.

Naves P, Bonifácio L, Sousa E. The pine wood nematode and its local vectors in the mediterranean basin. In: Paine DT, Lieutier F (eds). *Insects and diseases of mediterranean forest systems*. Springer, 2016, 329–378.

Oksanen J, Blanchet FG, Kindt R *et al*. vegan: Community Ecology Package. *R package version* 2.3-5 2015.

Oku H, Shiraishi T, Ouchi S *et al*. Pine wilt toxin, the metabolite of a bacterium associated with a nematode. *Naturwissenschaften* 1980;**67**:198–199.

Park D-S, Oh H-W, Jeong W-J *et al*. A culture-based study of the bacterial communities within the guts of nine longicorn beetle species and their exo-enzyme producing properties for degrading xylan and pectin. *J Microbiol* 2007;**45**:394–401.

Parks DH, Tyson GW, Hugenholtz P *et al*. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 2014;**30**:3123–3124.

Parte AC, LPSN-list of prokariotic names with standing in nomenclature. *Nucleic Acids Res* 2013;**42**:D613–D616.

Peel BL, Finlayson BL, McMahon TA. Updated world map of the Koppen-Geiger climate classification. *Hydrol Earth Syst Sc* 2007;**11**:1633–1644.

Pielou E. *Ecological Diversity*. Wiley 1996.

Pirttilä AM, Laukkanen H, Pospiech H *et al*. Detection of intracellular bacteria in the buds of scotch pine *(Pinus sylvestris* L.) by in situ hybridization. *Appl and Environ Microb* 2000;**66**:3073–3077.

Proença DN, Francisco R, Kublik S *et al*. The microbiome of endophytic, wood colonizing bacteria from pine trees as affected by pine wilt disease. *Scientific Reports* 2017 **7**:4205.

Proença DN, Francisco R, Santos CV *et al*. Diversity of bacteria associated with *Bursaphelenchus xylophilus* and other nematodes isolated from *Pinus pinaster trees* with pine wilt disease. *PloS One* 2010;**5**:e15191.

Proença DN, Grass G, Morais PV. Understanding pine wilt disease: roles of the pine endophytic bacteria and of the bacteria carried by the disease-causing pinewood nematode. *MicrobiologyOpen* 2016;**6**:e415.

Proença DN. *Role of endophytic microbial community in pine wilt disease*. Portugal: University of Coimbra, 2014.

Rawat SR, Männistö MK, Bromberg Y *et al*. Comparative genomic and physiological analysis provides insights into the role of *Acidobacteria* in organic carbon utilization in Arctic tundra soils. *FEMS Microbiol Ecol* 2012;**82**:341–55.

R Core Team. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. Austria, 2014.

Ricci I, Valzano M, Ulissi U *et al*. Symbiotic control of mosquito borne disease. *Pathog glob health* 2012;**106**:380–385.

Shannon C, Weaver W. *The Mathematical Theory of Communication*. Urbana: University of Illinois Press, 1964.

Sousa E, Bravo MA, Pires J *et al*. *Bursaphelenchus xylophilus* (Nematoda; Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal. *Nematology* 2001;**3**:89–91.

Sousa E, Vale F, Abrantes I. *Pine wilt disease in Europe: Biological interactions and integrated management*. Federação Nacional das Associações de Proprietários Florestais, 2015.

Tian B-Y, Cao Y, Zhang K-Q, Metagenomic insights into communities, functions of endophytes, and their associates with infection by root-knot nematode, *Meloidogyne incognita*, in tomato roots. *Sci Rep* 2015;**5**:17087.

Tiwari B, Manickam N, Kumari S, *et al*.. Biodegradation and dissolution of polyaromatic hydrocarbons by *Stenotrophomonas* sp. *Bioresour Technol* 2016;**2016**:1102–1105.

Vicente C, Espada M, Vieira P, Mota M Pine Wilt Disease: A threat to European forestry. *Eur J Plant Pathol* 2012a;**133**:89–99.

Vicente CSL, Ikuyo Y, Mota M *et al*. Pinewood nematode-associated bacteria contribute to oxidative stress resistance of *Bursaphelenchus xylophilus*. *BMC microbiol* 2013a;**13**:299.

Vicente CSL, Nascimento F, Espada M *et al*. Characterization of bacteria associated with pinewood nematode *Bursaphelenchus xylophilus*. *PloS One* 2012b;**7**:e46661.

Vicente CSL, Nascimento FX, Espada M *et al*. Characterization of bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis*, the insect vector of the pinewood nematode *Bursaphelenchus xylophilus*. *FEMS microbiol lett* 2013b;**347**:130–139.

Vicente CSL, Nascimento FX, Ikuyo Y *et al*. The genome and genetics of a high oxidative stress tolerant *Serratia* sp. LCN16 isolated from the plant parasitic nematode *Bursaphelenchus xylophilus*. *BMC Genomics* 2016;**17**:301.

Zas R, Moreira X, Ramos M *et al*. Intraspecific variation of anatomical and chemical defensive traits in Maritime pine (*Pinus pinaster*) as factors in susceptibility to the pinewood nematode (*Bursaphelenchus xylophilus*). *Trees-Struct Funct* 2014;**29**:663–673.

Zhao L, Mota M, Vieira P *et al*. Interspecific communication between pinewood nematode, its insect vector, and associated microbes. *Trends Parasitol* 2014;**30**:299–308.