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ORIGINAL ARTICLE

Improving reliability of PCR diagnostics for *Xylophilus ampelinus* by metagenome-informed circumscription of the target taxon

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Abstract

Xylophilus ampelinus is a xylematic bacterium causing bacterial blight of grapevine, a disease regarded as a potential threat for viticulture in several countries. Currently, PCR detection is pivotal in diagnostic protocols due to the bacterium's infrequent occurrence in the field and the technical advantages of PCR. Recent metagenomic studies have unveiled diversity in its taxonomic domain, unknown when the most widely used assays for the detection of *X. ampelinus* infections were developed. In particular, PCR assays relying on highly conserved sequence regions, such as those surrounding ribosomal RNA genes, may be substituted with more specific PCR assays. In this study, we first investigated the diversity of detectable grapevine endophytes related to but different from *X. ampelinus* and delineated the genotaxonomic boundaries of the species in relation to the known (meta)genomes of closely related bacteria. Then, by exploiting the wealth of genomes now available for bacteria classified in the *Burkholderiales*, we devised several sets of primers targeting only *X. ampelinus* and its closest relatives. These primers were employed to (i) genotaxonomically circumscribe the grapevine endophyte species related to but distinguishable from *X. ampelinus* and (ii) develop a robust multiplex PCR assay expected to be specific for the species *X. ampelinus* based on in vitro and in silico evidence. The adoption of the multiplex PCR assay presented here is expected to reduce the risk of false positives in the diagnosis of bacterial blight of grapevine.

KEYWORDS
bacterial blight, endophyte, grapevine

1 | INTRODUCTION

Xylophilus ampelinus has long been recognized as the sole species within the genus *Xylophilus*. The genus was established by Willems et al. (1987) to accommodate a plant-pathogenic bacterium previously named *Xanthomonas ampelina* (Panagopoulos, 1969). Hybridization studies between ribosomal RNA (rRNA) from *Xanthomonas campestris* and DNAs from different *Xanthomonas* species revealed that *Xanthomonas ampelina* was not related to the authentic *Xanthomonas* species (De Vos & De Ley, 1983). Based on this conclusion, *Xanthomonas ampelina* was transferred to the genus *Xylophilus*. The bacterium causes bacterial blight of grapevine, a disease originally described in Greece (Crete) (Panagopoulos, 1969) and successively reported with different names in France, Italy, Moldova,

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Portugal, Slovenia, Spain, South Africa and Japan (European and Mediterranean Plant Protection Organization [EPPO], 2009; Komatsu & Kondo, 2015). Presently, the bacterium is regulated as a quarantine pest in the regions of NAPPO (North American Plant Protection Organization) and IAPSC (Inter-African PhytoSanitary Council) and is included in the A2 list of pests recommended for regulation as quarantine pests by EPPO (Harrison et al., 2024).

Grapevine is the sole known host of this pathogen, which is locally transmitted via moist wind, rain, sprinkled irrigation (Bradbury, 1991) and shear blades during pruning and harvesting (Ridé et al., 1977). Studies exploiting *gfp*-marked strains showed that, after stem wounding and inoculation, the bacteria move downward through the xylem vessels to the crown, where they organize into biofilms (Grall & Manceau, 2003). Hence, propagative and planting material are the primary means of long-distance dissemination, stressing the importance of screening procedures for such materials in disease control and regulation.

Culturing *X. ampelinus* from diseased grapevine material onto medium is difficult (Serfontein et al., 1997), and no selective medium specific for *X. ampelinus* exists (Manceau et al., 2005). Serological methods have been developed (Gorris et al., 1989; Ridé et al., 1977), but their sensitivity and specificity are problematic (Manceau et al., 2005). Thus, given their rapidity, sensitivity, portability and easy implementation, PCR-based technologies are preferred for routine diagnostics. Within the European Horizon 2020 project VALITEST, Harrison et al. (2024) evaluated nine different assays for the diagnosis of *X. ampelinus*. Conventional PCR, real-time PCR, ELISA and immunofluorescence (IF) assays were considered in detail by different participant laboratories; the five PCR-based tests were found “to be fit for purpose” with similar performance and were widely preferred over immunological tests. In the EPPO protocol 7/96 (EPPO, 2009), the workflow concerning the diagnostic procedure for *X. ampelinus* recommends either isolation or two rapid tests on plant extracts based on different biological principles (IF, ELISA, conventional PCR or real-time PCR) as the initial step. Furthermore, the EPPO protocol suggests use of the PCR assay with XaTS1/XaTS2 primers (Manceau et al., 2005) and the real-time PCR-based method (Dreo et al., 2007). The use of two different biological principles (conventional PCR or real-time PCR in this case) mitigates the risk of false negatives. However, it doubles the risk of false positives and complicates the diagnostic procedure. Thus, there have been cases of commercial issues arising from the rejection of planting material, on the sole ground of positive results from one of the PCR assays, namely the ribosomal gene targeting assay using XaTS1/XaTS2 primers (Manceau et al., 2005). Given the evolutionary conservation of sequences, the reliability of diagnostic assays based on the ribosomal RNA genes is influenced by the diversity of the microbiome associated with the samples.

Over the last decade, there has been growing evidence that *X. ampelinus* is not an evolutionary isolated species, but rather belongs to a larger group of taxonomically related plant-inhabiting bacteria. Although only a limited number of these bacteria can be maintained and grown in vitro (Lee et al., 2020), metagenomic analyses (Yang & Iwasaki, 2014) suggested that the phytobiome may harbour an unexplored yet large taxonomic diversity laying within the boundaries of this genus. The finding of several grapevine samples that were positive to one recommended PCR assay (XaTS1/XaTS2; Manceau et al., 2005) but not to the other (Dreo et al., 2007) prompted us to perform a systematic study to obtain a clearer definition of the taxonomic specificity, essential for a reliable and improved PCR diagnostic assay specific for *X. ampelinus*.

2 | MATERIALS AND METHODS

2.1 | Samples and strains

DNA samples and the bacterial strains used in this work, including 11 strains of *X. ampelinus* isolated from different geographic areas and grapevine cultivars and five strains of the related genera *Paracidovorax*, *Acidovorax* and *Variovorax*, are listed in Table S1. Samples of grapevine and other plants were collected during the monitoring of grapevine orchards and vineyards initiated by the Plant Protection Service of the Friuli Venezia Giulia region.

2.2 | Isolation trials

All isolation attempts were performed on asymptomatic material that had previously tested positive to the primer pairs XaTS1/XaTS2 (Manceau et al., 2005) and negative to the primers Xamp14F/Xamp104R (Dreo et al., 2007). One gram of petioles was collected from different leaves and disinfected in a plastic bag with hydrogen peroxide for 30 s under a laminar flow hood (sterile conditions). The samples were then rinsed with sterile water and placed in an extraction bag (Bioreba Universal, 12 × 15 cm). Five millilitres of sterile phosphate-buffered saline (PBS) added to the extraction bag, and the samples were homogenized with a semi-automated Texor homogenizer (Lavorazioni Meccaniche Linzi Mauro) for 60 s.

The crude extracts were then diluted 1/10 and 1/100 in nutrient broth (NB; Biolife Italiana) under sterile conditions. One hundred microlitres of the crude and diluted extracts were plated onto the surface of at least two (per dilution) freshly prepared and appropriately labelled Petri dishes containing nutrient agar (NA; Biolife Italiana). The plates were incubated at 25°C until colony appearance (1–2 weeks).

2.3 | Nucleic acid extraction and diagnostic PCR testing

Pure bacterial cultures were grown in NB with agitation. After 24 h, genomic DNA (gDNA) was extracted from 1 mL of culture using a Wizard DNA purification kit (Promega) following the manufacturer's instructions. DNA was measured and checked for quality using a spectrophotometer (NanoDrop).

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The mixed bacterial cultures, recovered from the attempts to grow plant endophytes in axenic culture, were suspended in buffer, and DNA was extracted using the QIAamp DNA Mini kit (Qiagen), in accordance with the manufacturer's instructions.

From plant tissues, DNA was extracted from 1 mL of homogenate in PBS. After centrifugation (13,000 *g* for 10 min at 4°C), the pellet was resuspended in 700 μL of warm 2% CTAB extraction buffer containing 1 μL of proteinase K (600 mAU/mL) and incubated for 30 min at 60 ± 1°C in a thermostatic bath. Then, the homogenate was centrifuged (13,000 *g* for 15 min) and the supernatant (500 μL) was transferred to a new tube. Bacterial DNA was extracted using the Qiasymphony Mericon Bacteria kit (Qiagen) and the Automated Qiasymphony SP instrument (Qiagen).

PCR assays with XaTS1/XaTS2 (Manceau et al., 2005) and Xamp14F/Xamp104R (Dreo et al., 2007) primer pairs were carried out as described by the authors and according to the internal method developed by the official laboratory of the Plant Protection Service of Friuli Venezia Giulia region (Italy) and Dreo et al. (2007), respectively. The method, accredited under ISO 17025, is based on a multiplex real-time PCR for the detection of a grapevine reference gene (chaperonin; Angelini et al., 2007) and of *X. ampelinus*, using XaTS1/XaTS2 and Xamp14F/Xamp104R primer pairs.

A specific TaqMan probe internal to the amplicon obtained with the XaTS1/XaTS2 primers (XAMP-ManP: 5′ HEX-ACAGCCGATTGATCGAACAAT-MGB 3′) was designed to be used in multiplex and singleplex real-time PCR assays. Specificity and sensitivity of the probe were evaluated in the validation project, necessary to the accreditation of the internal method under ISO 17025.

The estimated limit of detection (LOD) of the internal method was 15 copies of genomic DNA with XaTS1/XaTS2 primers (Manceau et al., 2005) and 10 copies of genomic DNA with Xamp14F/Xamp104R primers (Dreo et al., 2007). The cut-off of the method was established at 37 C_q for XaTS1/XaTS2 primers and at 38 C_q for Xamp14F/Xamp104R primers.

To synthesize amplicons for Sanger sequencing, PCRs were carried out with GoTaq Flexi DNA polymerase (Promega) using the conditions recommended by the manufacturer and using 1 μL of target DNA solution (2–20 ng/μL). The primers tested are highlighted in the list in Table S2. The PCR programme consisted of 94°C for 2 min; followed by 40 cycles of at 94°C for 40 s, 30 s at 60°C, and 72°C for 40 s; then 72°C for 8 min.

Selected amplicons were purified using the Gene JET PCR purification kit (Thermo Fisher) and sent to BMR Genomics (Padua, Italy) for Sanger sequencing.

2.4 | Third-generation sequencing

For whole DNA analysis, an Oxford Nanopore Technology (ONT) library was generated using 1 μg of high molecular weight (HMW) gDNA from each sample. This was prepared following the native barcoding genomic DNA protocol using Ligation Sequencing (SQK-LSK109) and Native Barcoding Expansion 1–12 (EXP-NBD104) kits. The library pool generated contained, on average, 117 ng DNA (from plant and mixed culture samples) or 12 ng DNA (from *X. ampelinus* strains). Adapter ligation was performed with the short fragment buffer. The library was then sequenced on a MinION (ONT) device equipped with an R9.4.1 nanopore cell according to manufacturer's instructions.

For the library preparation and the sequencing of long amplicons using ONT, the Native barcoding amplicons protocol (including EXP-NBD104 and SQK-LSK110 kits) was used. Primer pairs nano1f/nano1r (nano1f: 5′-TTTCTGTTGGTGCTGATATTGCTCTTCGGAACTAGTGCTTGC-3′, position 5470 in accession NZ_JAMOFZ010000042.1; nano1r: 5′-ACTTGCCTGTCGCTCTATCTTCCGCCCTACTTGTCGTTAACTT-3′ position 2829) and nano2f/nano2r (nano2f: 5′-TTTCTGTTGGTGCTGATATTGCCGTCGATCTGTAGCTGGTCT-3′ position 5098; nano2r: 5′-ACTTGCCTGTCGCTCTATCTTCCTACGGCTTCCCTATTCGGT-3′ position 2584) were specifically designed around the XaTS1/XaTS2 region. PCR solutions contained 1× Phusion high-fidelity buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each primer, 0.02 U/μL of Phusion high-fidelity DNA polymerase and 2 μL of DNA (2–20 ng/μL). The PCR programme consisted of 98°C for 30 s; followed by 40 cycles of 98°C for 10 s, 30 s at 63°C (nano1f/nano1r) or 68°C (nano2f/nano2r), and 72°C for 80 s; then 72°C for 7 min. The library pool was generated containing an average of 84 ng of each prepared amplicon.

2.5 | Sequence analysis and primer design

The sequences from MinION runs of the genomic libraries were assembled with Canu (Koren et al., 2017) and annotated with Rast (Aziz et al., 2008). Using OMA-standalone (Altenhoff et al., 2019) for orthologous group building, the putative proteins were compared with those predicted from 18 genomes, including 12 from strains/metagenomes deposited in the NCBI database as belonging to the genus *Xylophilus*, four from strains of the related genera *Paracidovorax* and *Variovorax* for reference, and two genomes obtained in this work (Table S1). Next, 138 orthologous groups containing one putative protein for each strain were selected and then aligned using ClustalW2 (Larkin et al., 2007). The DNA sequences encoding the putative proteins were retrieved using a custom script and aligned. Alignments were used to build trees and networks with the aid of Seaview (Gouy et al., 2010) and SplitsTree (Huson & Bryant, 2005).

Generation of genome-wide PCR primer sequences was carried out using QuantPrime (Arvidsson et al., 2008; available at https://quantprime.mpimp-golm.mpg.de/), following inclusion of the genome of *X. ampelinus* CECT 7646 within the list of the exploitable genomes, courtesy of the authors. The primer specificity was determined using recursive highly stringent BLASTn (Altschul, 1997) searches in a custom database of the 5878 genome assemblies that had been deposited in NCBI and obtained from bacteria belonging to the *Burkolderiales*.

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2.6 | Multiplex PCR diagnostic assay

The multiplex PCR for improved diagnosis of *X. ampelinus* proposed in this paper was prepared in a 25 μL volume: 12.5 μL 2× KAPA HiFi HotStart ReadyMix, 0.25 μL of each 20 μM solution of primers XaWP4F (5′-TCCTGTGGTTTCGGCCTTGTTG-3′)/XaWP4R (5′-TCGAGCAGCTTGGCCGATTTAG-3′) (801 bp), 0.375 μL of each 20 μM solution of primers XaWP5F (5′-GGCGCTGCAACAAATGCTATCG-3′)/XaWP5-R (5′-AGCTTTCGCTGGTTCTGCGTTG-3′) (983 bp), Xamp20F1 (5′-ATTCGTCATCCAGACGCTCG-3′)/Xamp20R1 (5′-ACCAGATCCAGCAAGAGTGC-3′) (317 bp), 1 μL target DNA solution (2–20 ng/μL) and 9.5 μL pure water. The PCR programme consisted of 95°C for 3 min; 32 cycles at 98°C for 20 s, 20 s at 68°C, and 72°C for 1 min.

3 | RESULTS

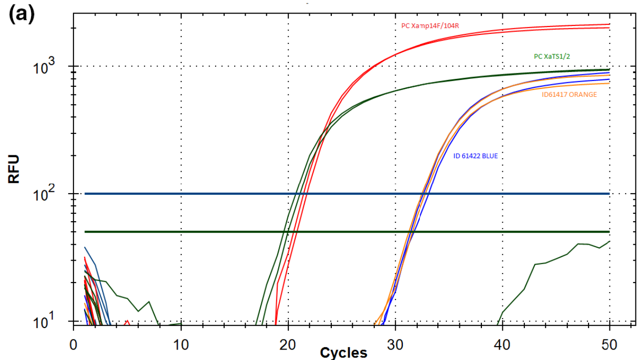
3.1 | Clarification of the inconsistent results in PCR diagnostics

During routine sampling and testing for certification purposes, symptomless grapevine samples were found that were positive to only one of the two diagnostic tests for the causal agent of grapevine bacterial blight. Attempts to isolate an *X. ampelinus*-related strain on agar plates repeatedly failed, although the bacteria were able to grow in mixed cultures in vitro. According to the real-time PCR results (Figure 1a,b), for some samples, the targeted DNA fragment was 10- to 100-fold more abundant in mixed cultures than in plant DNA.

The DNA samples extracted from the mixed cultures and from their source plants were used as templates for PCR amplification with primers spanning more than 2000 bp on both sides of the XaTS1/XaTS2 129 bp amplicon, and the resulting amplicons were Nanopore sequenced. The obtained data were mined for reads with sequence similarity to the target sequence and the primers of the XaTS1/XaTS2 PCR assay (Manceau et al., 2005). The detected reads were aligned with the homologous regions of reference genomes and used to build the dendrogram shown in Figure 2. Accordingly, the selected Nanopore-sequenced reads revealed the presence in the samples of targets originating from organisms strictly related to, yet not identical to, *X. ampelinus* reference strains. However, given the error rate of MinION sequencing, the lack of knowledge of diversity within the species, and the conservation of the genomic region, the results cannot be regarded as conclusive in addressing the identity of the source of the PCR-positive signals.

3.2 | Genome-wide analysis of the genus *Xylophilus*

Having assessed that some grapevine samples contain nucleic acids that may act as target of PCR amplifications with the XaTS1/XaTS2 PCR assay (Manceau et al., 2005), we focused on establishing whether such target sequences should be considered as belonging to new strains of the species *X. ampelinus*. To this end, we established a



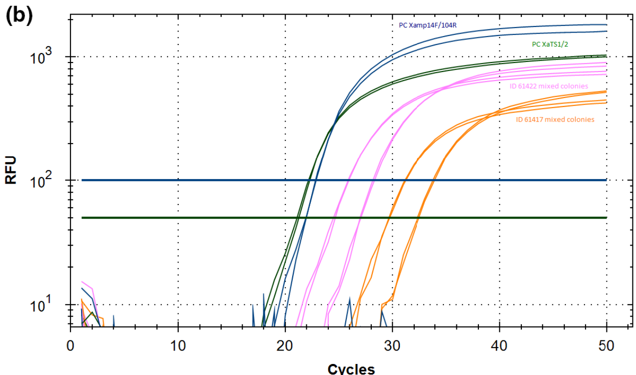


FIGURE 1 Plot of multiplex real-time PCR amplification of DNA extracted from (a) grapevine plants and from (b) mixed bacterial cultures of plant endophytes. Curves shown in (a) resulted from the amplification of the positive control (PC) of *Xylophilus ampelinus* CFBP 2061 (Xamp14F/Xamp104R primers in red, XaTS1/XaTS2 primers in green), DNA ID 61417 extracted from grapevine (in orange, primers XaTS1/XaTS2), and DNA ID 61422 extracted from *Corylus avellana* (in blue, primers XaTS1/XaTS2). Curves shown in (b) resulted from the amplification of positive control (PC) of *X. ampelinus* CFBP 2061 (Xamp14F/Xamp104R primers in blue, XaTS1/XaTS2 primers in green), mixed cultures obtained after two different isolation trials of sample ID 61422 (in fuchsia, primers XaTS1/XaTS2), and from two different isolation trials of sample ID 61417 (in orange, primers XaTS1/XaTS2). RFU, relative fluorescence units.

small collection of *X. ampelinus* isolates for use in genome sequencing and PCR assays, as described below. To better define the genotaxonomic domain corresponding to the species *X. ampelinus*, the genome drafts of two strains (CFBP 5787 and CFBP 4864) were obtained by Nanopore sequencing and compared with genomes available from public databases. In detail, we carried out a homologue inference using OMA, which identified 11,672 orthologous groups and selected the complete groups, that is, those that include one member gene for each genome. The clustering based on protein sequence similarity (Figure S1) supported the results previously reported by Portier et al. (2022): the genomes of the culturable strains of *X. ampelinus* isolated from grapevine are a homogeneous group well distinct from other genomes (from cultured strains or metagenome) originated from other plants or environments and deposited as *Xylophilus* sp. or even *X. ampelinus* in public databases. Moreover, Figure 3 shows a consensus (strict) tree of 138 individual trees (method = count, threshold = 90%) and a clade genotaxonomically

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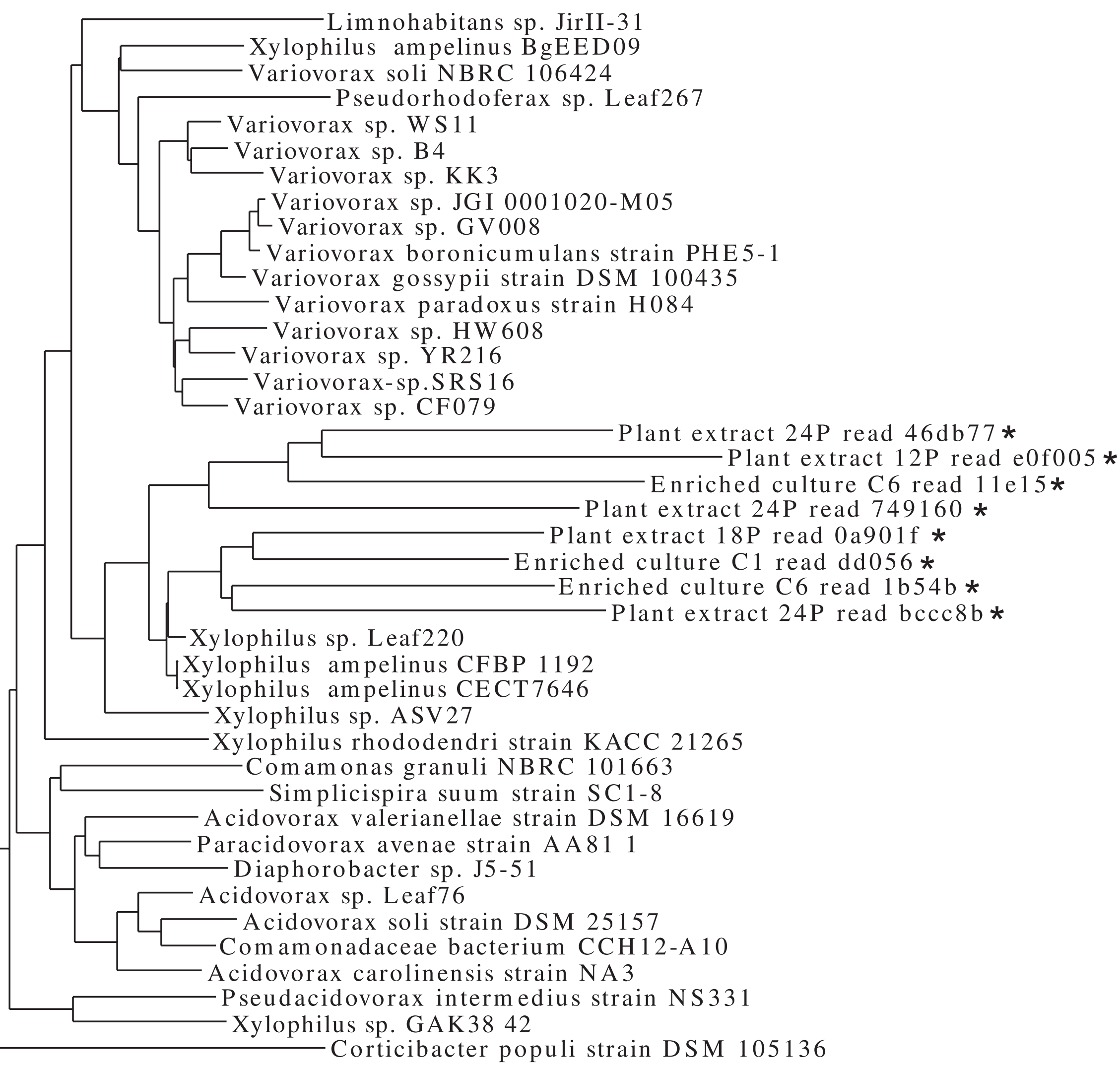

FIGURE 2 Neighbour-joining tree, based on Jaccard's similarity, of sequences of reference organisms and individual long reads obtained by Nanopore sequencing of PCR amplification of extracts from grapevine plants (P) and from mixed bacterial cultures resulting from isolation attempts (C). Sequences obtained in this work are marked with '*'.

well distinct from other entities, referred to as *X. ampelinus* sensu stricto. In particular, it is worth noting that in >90% of the trees, the genome of an isolate obtained from an *Arabidopsis thaliana* leaf, named *Xylophilus* sp. strain Leaf220 (Bai et al., 2015), is not in the *X. ampelinus* sensu stricto clade. These results support the notion that there are several bacteria similar to *X. ampelinus* that behave as endophytes of plants. Considering the ratio of genomes from metagenomic studies versus genomes of cultured isolates, it is reasonable to speculate that a large fraction does not (easily) grow in vitro.

3.3 | Characterization of grapevine endophytic bacteria

With the aid of the web tool QuantPrime (Arvidsson et al., 2008) and custom scripts, we generated a genome-wide collection (1273 genes targeted) of PCR primer pairs and checked their potential target matches against an ad hoc database built using 5000 genomes of bacteria belonging to the *Burkolderiales*. According to the results, the primer pairs were ordered (Table S2) based on their taxonomic specificity. Then, we selected two sets of primer pairs: a first set was chosen with loose specificity to allow the amplification of DNA fragments from organisms belonging to *Xylophilus* and closely related taxa. The second set was chosen with strict specificity to *X. ampelinus* sensu stricto and was used to design a disease diagnostic assay (see Section 3.4).

The first set of primer pairs was used to PCR amplify *Xylophilus*-related DNA fragments from DNA extracts of plant samples and from the mixed cultures resulting from isolation attempts. With some primer pairs, due to broader specificity, the amplicons contained mixed target amplified fragments (not shown). We exploited RFLP analysis to pinpoint fragments consistently

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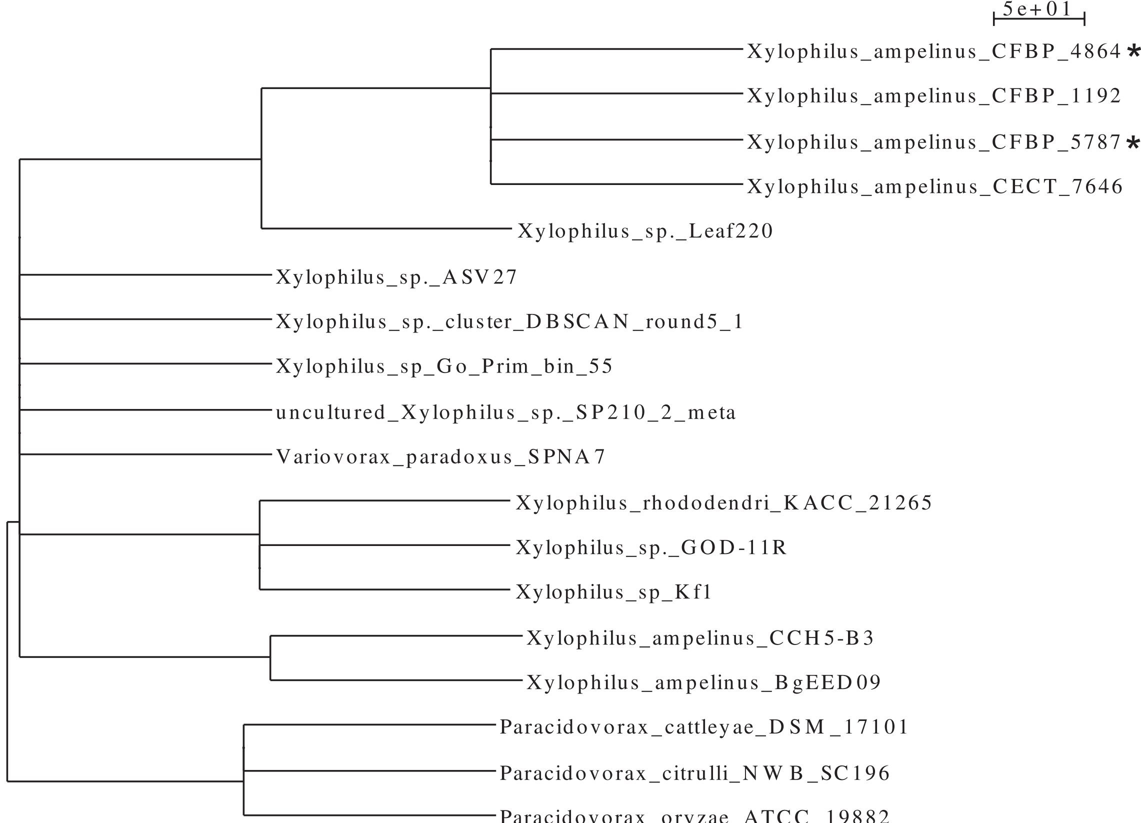

FIGURE 3 Strict (only branches supported by 90% of the trees are drawn) consensus of 138 neighbour-joining trees constructed on alignments of orthologues from genomes deposited in the NCBI database as bacteria belonging to *Xylophilus* spp. and selected related species. Genomes obtained in this work are marked with '*'.

amplified and with the lowest degree of sequence diversity, and we selected them for sequencing. The DNA sequencing of amplified DNA (primer pairs designed on accessions WP_110464228 and WP_110464371) from grapevine plant samples provided sequences of high quality without ambiguities. These sequences could be aligned with the homologous regions from the genomes of *X. ampelinus* and related species and metagenomes. The resulting dendrograms (Figure 4) provided evidence of the presence of bacteria that are more closely related to the *A. thaliana* endophyte *Xylophilus*. sp. Leaf220 (Bai et al., 2015) than to the clade made of the bacterial isolates from grapevine that are the cause of the bacterial blight, that is, *X. ampelinus* sensu stricto.

Moreover, exploiting the real-time PCR results on total DNA extracted from plants, we selected four samples for analysis by direct Nanopore sequencing. Mining of resulting reads provided further sequences homologous to the investigated regions. A very long read (33,568 nucleotides comprising about 30 open reading frames [ORFs]) was found and showed perfect synteny with the genomes of *X. ampelinus* sensu stricto and of the *A. thaliana* endophyte *Xylophilus* sp. Leaf220. The percent identity calculated from the alignment was 88% versus *X. ampelinus* sensu stricto and 90% versus the *A. thaliana* endophyte *Xylophilus* sp. Leaf220. Twenty-two ORFs were extracted and compared with the orthologous genes in the genomes deposited in the public databases as *Xylophilus* spp. and reference genomes, resulting in the consensus tree (median) shown in Figure 5. The results of these analyses consistently support the notion that the Nanopore-sequenced read was not part of the *X. ampelinus* sensu stricto clade but was more similar to the *A. thaliana* endophyte *Xylophilus* sp. Leaf220.

In conclusion, the endophytic bacterial community detected from plant extracts and the mixed cultures contains bacteria that are genotaxonomically more similar to members of the genus *Xylophilus* than to any other recognized genus. These bacteria can be detected by the XaTS1/XaTS2 PCR diagnostic test (Manceau et al., 2005), but do not belong to the taxon *X. ampelinus* sensu stricto.

3.4 | Improved diagnostic PCR for *X. ampelinus*

As mentioned above, QuantPrime (Arvidsson et al., 2008) and custom scripts allowed us to identify and test in silico several primer pairs with high specificity to *X. ampelinus* sensu stricto. The primer pairs were also tested in vitro by PCR using our collection of *X. ampelinus* strains and DNA extracts that gave positive results in the XaTS1/XaTS2 PCR assay (Manceau et al., 2005). Based on the results of this screening, we ultimately designed and tested a diagnostic

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0.02

Hydrogenophaga palleronii NBRC 102513
Xylophilus ampelinus BgEED09
Variovorax soli NBRC 106424
Variovorax paradoxus S110
Variovorax boronicumulans NBRC 103145
Xylophilus rhododendri KACC 21265
Xylophilus ampelinus CFBP 1192
Xylophilus ampelinus CECT 7646
Xylophilus ampelinus CFBP 1192
Xylophilus ampelinus CFBP 5787 *
Plant extract 17P *
Plant extract 13P *
Plant extract 15P *
Plant extract 12P *
Plant extract 25P *
Plant extract 23P *
Xylophilus sp. Leaf220
Plant extract 14P *
Plant extract 18P *
Polaromonas jejuensis NBRC 106434
Delftia acidovorans ATCC 11299b
Delftia nitroreducens DSM 15985
Paracidovorax cattleyae 301576
Paracidovorax cattleyae strain DSM 17101
Paracidovorax citrulli DSM 1070
Paracidovorax oryzae ATCC 19882
Paracidovorans wautersii DSM 27981

0.05

Variovorax paradoxus NBRC 15149
Variovorax paradoxus S110
Variovorax gossypii DSM 100435
Variovorax boronicumulans NBRC 103145
Variovorax soli NBRC 106424
Xylophilus rhododendri KACC 21265
Delftia acidovorans ATCC 11299b
Hydrogenophaga intermedia DSM 5680
Delftia nitroreducens DSM 15985
Acidovorax soli DSM 25157
Polaromonas jejuensis NBRC 106434
Curvibacter delicatus NBRC 14919
Hydrogenophaga palleronii NBRC 102513
Acidovorax temperans DSM 7270
Xylophilus sp.. ASV27
Xylophilus ampelinus CFBP 1192
Xylophilus ampelinus CFBP 5787 *
Xylophilus ampelinus CFBP 1192
Xylophilus ampelinus CECT 7646
Plant extract 16P *
Plant extract 18P *
Plant extract 13P *
Plant extract 14P *
Plant extract 25P *
Plant extract 17P *
Plant extract 15P *
Plant extract 23P *
Plant extract 12P *
Xylophilus sp.. Leaf220
Plant extract 24P *
Paracidovorans wautersii DSM 27981
Paracidovorax citrulli DSM 1070
Paracidovorax cattleyae strain DSM 17101
Paracidovorax cattleyae 301576
Paracidovorax oryzae ATCC 19882
Paracidovorax avenae ATCC 19860

FIGURE 4 Neighbour-joining tree based on Jaccard's similarity of sequences of two regions (left: Fragment #19; right: Fragment #20) obtained from extracts of grapevine samples amplified with primers of broad specificity (marked with '*') and the homologous regions in the genomes of reference strains of *Xylophilus ampelinus* and related bacterial species and metagenomes.

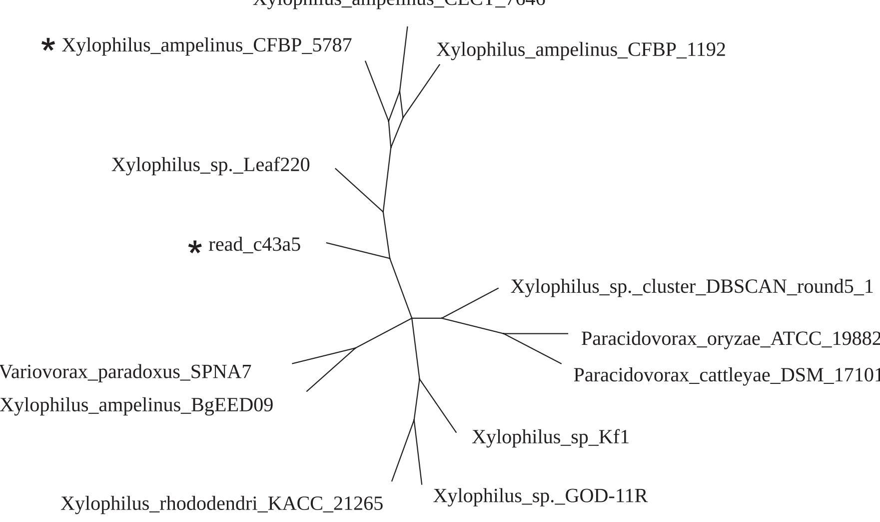

FIGURE 5 Consensus network of 22 trees constructed on the alignment of 22 open reading frames of the Nanopore read c43a5, obtained from total DNA extracted from grapevine, and their orthologous genes in reference genomes of *Xylophilus* spp. and other bacteria. Threshold = 0.5; EdgeWeights = mean; the lengths of the branches connecting the same nodes were averaged and drawn if present in at least 50% of the trees. Data obtained in this work are marked with '*'.

multiplex PCR with three highly specific primer pairs targeting three different genes of *X. ampelinus* sensu stricto. The predicted sizes of the amplicons were 317 bp (Xa20F1/R1), 801 bp (XaWP #4-F/R) and 983 bp (XaWP #5- F/R).

The primer set was specific when tested with two distinct PCR chemistries, namely GoTaq Flexi DNA polymerase and KAPA HiFi HotStart. Both PCR tests were reliable and KAPA HiFi HotStart provided the lowest limit of detection (5 pg, Figure 6).

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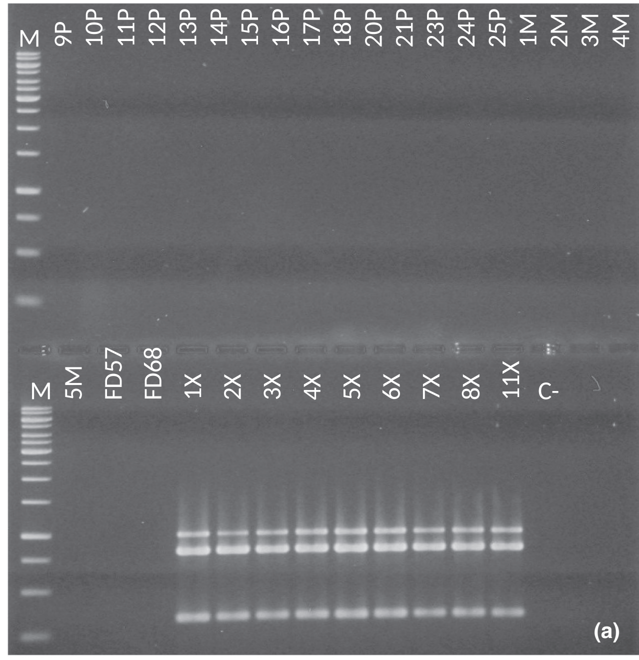

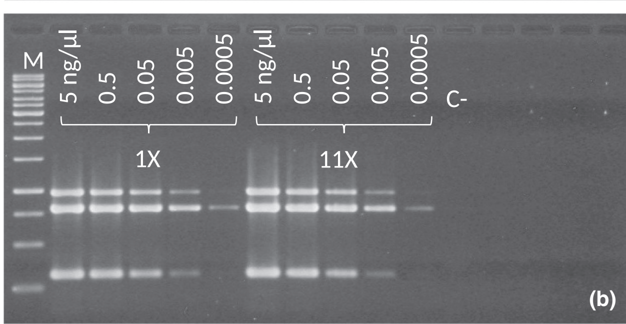

FIGURE 6 Agarose (1.5%) gel electrophoresis of the multiplex PCR diagnostic for *Xylophilus ampelinus* sensu stricto. (a) Specificity of multiplex assay for *X. ampelinus*; grapevine DNA extracts that had reacted positively with the current diagnostic primers XaTS1/XaTS2 (Manceau et al., 2005) are indicated with P, bacterial DNA extracts of *X. ampelinus* are indicated with X and those of closely related species are indicated with M (Table S1); FD57 and FD68 are DNA extracts from grapevine infected with flavescence dorée. (b) Sensitivity of multiplex assay for *X. ampelinus*; five serial dilutions (1:10) of samples 1X and 11X *X. ampelinus* genomic DNA (from 5 to 0.0005 ng/µL), diluted with healthy grapevine DNA solution (20 ng/µL). M: GeneRuler 1 kb DNA Ladder (Thermo Scientific), bottom to top: 250–10,000 bp; C−: negative control devoid of DNA template.

4 | DISCUSSION

Despite the absence of recent outbreaks and the limited worldwide phytopathological impact of grapevine bacterial blight, the diagnosis of its causative agent, *X. ampelinus*, remains relevant due to associated commercial and quarantine issues. Recently, Portier et al. (2022) highlighted the significant genomic homogeneity of the *X. ampelinus* species based on the results of the analysis of *gyrB* and *rpoD* gene sequences in 93 strains and the whole genome sequencing of one strain. In this work, we provided the genome sequences of two additional strains and the results of further analyses that confirmed and supported their findings.

According to Portier et al. (2022), the MetaMetaDB (Yang & Iwasaki, 2014) hits that match *X. ampelinus* 16S rDNA with 97% sequence identity are found across a variety of ecosystems, such as beetle, soil, rhizosphere, marine, freshwater and others. The authors argued that the actual occurrence of this species in the environment is probably underestimated. It may be now worth considering that the occurrence of the corresponding 16S rDNA type rather than the pathogen may be underestimated. Indeed, the genome-wide analysis conducted in the present study revealed that the well-defined genotaxonomic clade comprising strains of the plant-pathogenic bacteria causing grapevine blight is not precisely defined by its 16S rDNA type. Other bacteria, sharing more than 97% 16S rDNA sequence identity with this clade, may be discernible for their genomic features as well as for traits determining their ability to be grown in vitro or to be pathogenic on grapevine. The occurrence of pathogenic and nonpathogenic strains among closely related bacteria is not uncommon to phytopathology. Even for the widespread *Pseudomonas syringae*, originally identified as a pathogen, it has since been found that many isolates belonging to the species are nonpathogenic and exist as commensals on plants (Xin et al., 2018).

In this study, we present evidence from several asymptomatic grapevine field samples of a taxon that is genotaxonomically distinct from *X. ampelinus* sensu stricto, with unknown phytopathogenic characteristics. An effective diagnosis of the agent of grapevine bacterial blight should rely on diagnostic assays that react positively only with DNA from the genotaxonomically homogeneous and well-defined *X. ampelinus* sensu stricto clade, and not with DNA from entities outside this clade. Therefore, the diagnostic assay should preferably be based on non-ribosomal DNA sequences. To address this need, we developed a new, robust multiplex PCR assay, utilizing the specificity of three unrelated regions of the genome of the target bacterial taxon. This new, single assay provides simultaneous testing for the presence of three different diagnostic regions of the *X. ampelinus* genome that, as we showed, are more specific than the ribosomal DNA region targeted by the conventional PCR reported in the protocol 7/96 (EPPO, 2009). The adoption of the multiplex PCR assay presented here is therefore expected to minimize false positives in the diagnosis of bacterial blight of grapevine. While the assay demonstrated high reliability in testing pure bacterial cultures and a variety of plant extracts, further extensive testing with field samples is necessary before proposing its inclusion in certification protocols.

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DATA AVAILABILITY STATEMENT

Data generated in this study are available in part as article supplementary material and in part in the European Nucleotide Archive at www.ebi.ac.uk/ena/ as project PRJEB75643.

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