Differentiation of the Xanthomonas hortorum – Xanthomonas hydrangeae Species Complex Using Sensitive and Selective LAMP Assays

Nay C. Dia1,2, Bart Cottyn3, Jochen Blom4, Theo H. M. Smits1 and Joël F. Pothier1*

1 Environmental Genomics and Systems Biology Research Group, Institute for Natural Resource Sciences, Zurich University of Applied Sciences (ZHAW), Wädenswil, Switzerland, 2 Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, Zürich, Switzerland, 3 Plant Sciences Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Merebeke, Belgium, 4 Bioinformatics and Systems Biology, Justus-Liebig University Giessen, Giessen, Germany

The seven pathovars of Xanthomonas hortorum and Xanthomonas hydrangeae, referred to as the X. hortorum – X. hydrangeae species complex, cause disease on a multitude of plants, including crops, ornamental and wild plants. Cross-pathogenicity was proven for some of the strains within this species complex. It is thus important to have highly specific and fast diagnostics methods for members of the X. hortorum – X. hydrangeae species complex. A comparative genomic analysis was conducted for representative members within the complex to identify singletons for use as genomic targets for the assays. Seven loop-mediated isothermal amplification (LAMP) diagnostics assays were developed for the detection of six clades within the X. hortorum – X. hydrangeae species complex, in addition to one assay specific for the entire species complex. Primer sets were tested on a set of 62 reference strains. The primer sets amplified their respective targets within 15 minutes. Based on the reference set, all assays had a sensitivity, specificity, and efficiency of 100%. The assays were used on a validation set of 60 strains. According to the LAMP results, out of the 60 strains, 39 strains were assigned to one of the clades within the complex, 9 were assigned to the complex but to yet undefined clades within the complex, and 12 strains were previously misclassified as X. hortorum since their genomic DNA did not yield amplification with any of the assays. The seven genome-based assays are promising for use as diagnostic tools for various members within the X. hortorum – X. hydrangeae species complex, and for assigning new and historical isolates to this complex.

Keywords: singletons, bacterial leaf spot, diagnostic methods, natural infection, hydrangea
INTRODUCTION

Diagnostic methods are an essential part of integrated pest management programs, especially for the control of bacterial spot diseases (Agrios, 2005; Janse, 2005) such as the ones caused by Xanthomonas hortorum and Xanthomonas hydrangeae (Dia et al., 2021; Dia et al., 2022). Robust, field-deployable methods, with minimal sample preparation and post-amplification handling, are valuable tools for in-field or point-of-entry molecular detection of plant pathogens (Donoso and Valenzuela, 2018). Field-deployable methods, such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), are effective methods that are of interest in diagnostic procedures (Donoso and Valenzuela, 2018; Catara et al., 2021). These two isothermal methods rely on strand displacement by the polymerase, and exponentially amplify the target regions at a constant temperature (Notomi et al., 2000; Piepenburg et al., 2006). Results can be detected visually by turbidity or color change (Mori et al., 2004; Tomita et al., 2008), or at end-point amplification by gel electrophoresis, or alternatively in real-time by measuring fluorescence (Bühlmann et al., 2013; Getaz et al., 2017; Ruinelli et al., 2017). Both LAMP and RPA have been previously used for the detection of plant pathogens, including those within the X. hortorum – X. hydrangeae species complex, such as X. hortorum pathovars (pv.s) gardneri, carotae and pelargonii (Temple and Johnson, 2009; Temple et al., 2013; Strayer-Scherer et al., 2019; Stehlíková et al., 2020; Dia et al., 2021).

The X. hortorum – X. hydrangeae species complex currently comprises seven pathovars of X. hortorum (Morinière et al., 2020) and the recently described novel species X. hydrangeae (Dia et al., 2021). The species X. hortorum includes the devastating bacterial pathogen of geranium (X. hortorum pv. pelargonii) (Munnecke, 1954; Nameth et al., 1999), the internationally regulated seed-borne pathogen affecting carrot (X. hortorum pv. carotae) (Scott and Dung, 2020), the pathogen present in most lettuce-growing regions (X. hortorum pv. vitians) (Sahin, 1997; Morinière et al., 2020), and the pathogen which, in addition to three other Xanthomonas pathogens, causes bacterial spot of tomato and pepper (X. hortorum pv. gardneri) (Jones et al., 2004; Osdaghi et al., 2021). The three remaining pathovars of X. hortorum (pv.s taraxaci, hederae and cynarae) cause, respectively, bacterial spot and/or bacterial blight on dandelion (Niederhauser, 1943), ivy (Arnaud, 1920; White, 1934) and artichoke (Ridé, 1956; Trébaol et al., 2000; Timilsina et al., 2019). X. hydrangeae causes bacterial leaf spot on Hydrangea (Dia et al., 2021). Strains within the species complex affect 65 plant species spread over 15 botanical families (Dia et al., 2022).

The goal of this study was to develop specific and rapid genomics-based LAMP assays targeting six X. hortorum – X. hydrangeae clades, in addition to one assay detecting the totality of the complex. Assay metrics (e.g., sensitivity, specificity, and efficiency) were assessed based on the genomic DNA of reference strains (n = 62). The assays were used to differentiate various xanthomonads (e.g., unclassified or with an uncertain classification) obtained from culture collections and diagnostic laboratories. Furthermore, two assays, targeting the species complex and X. hydrangeae, were used to diagnose X. hydrangeae on Hydrangea spp. with typical leaf spots, showing the direct application of the developed assay.

MATERIALS AND METHODS

Bacterial Strains, Bacterial Boiled Cells, Bacterial Genomic DNA, and Plant Extracts Preparation

Bacterial strains were stored in a 1:1 (v:v) NYGB and 100% glycerol at -80°C, and were revived from glycerol stocks. Strains were cultivated on nutrient-yeast glycerol agar (NYGA, 5 g L⁻¹ peptone, 3 g L⁻¹ yeast extract, 20 g L⁻¹ glycerol and 15 g L⁻¹ agar) or in NYG broth overnight or for two days at 28°C (with shaking at 200 rpm). Cells grown overnight in NYGB were used to prepare boiled cells or to extract genomic DNA.

Boiled cells were prepared by transferring one loop of fresh bacterial cells into 1 ml of ddH₂O, followed by boiling at 98°C for 15 minutes and dilution at 1:100 with ddH₂O. To obtain high-quality DNA, bacterial genomic DNA was extracted from overnight cultures with the NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The genomic DNA was checked using a Q5000 spectrophotometer (Quawell, San Jose, CA). Genomic DNA amounts were normalized to 1 ng µl⁻¹ based on spectrophotometer values, and the normalized concentration of random samples was checked using Quanti-it PicoGreen double-stranded DNA quantification assay (Thermo Fisher Scientific, Waltham, MA).

Plant material was prepared as follows: two fresh 1 cm-diameter punches from leaves of Hedera helix, Taraxacum officinale, Lactuca sativa, Pelargonium sp. and Hydrangea macrophylla, and 50 mg (± 3 mg) of Daucus carota seed were placed in tubes containing steel beads (OptiGene Ltd, Horsham, United Kingdom). For spiking the template with plant extracts, the plant material was bead-beaten using the plant lysis kit according to the manufacturer’s protocol (OptiGene Ltd). The prepared plant material was flash-frozen in liquid nitrogen and stored at -80°C for subsequent use.

Genome-Informed Target Identification

Target identification was based on the whole-genome phylogeny of the X. hortorum – X. hydrangeae species complex (Dia et al., 2021). Genomes of 17 X. hortorum – X. hydrangeae species complex strains and three outgroup strains (Xanthomonas arboricola pv. juglandis CFBP 2528, Xanthomonas populi CFBP 1817 and Xanthomonas campestris pv. campestris ATCC 33913) (Table 1) were submitted to the comparative genomics platform EDGAR v3.0 (Dieckmann et al., 2021). Genome completeness was analyzed based on 1,152 core genes using BUSCO v5.2.2 (Manni et al., 2021) using xanthomonadales_odb10 (2020-03-06), the mode “genome” and dependencies hmmsearch v3.1 and prodigal v2.6.3. BUSCO scores were all ≥ 97%. Potential genome contamination was assessed using ContEst16S (Lee et al., 2017). The 16S rRNA
sequences were intact for all genomes except for five draft genomes, for which the integrity check was inconclusive as only one 16S rRNA copy was found.

The phylogeny of the 20 genomes was built in the EDGAR platform, based on a core genome of 2,345 genes per genome. There was a total of 831,037 amino acid residues per genome. Each of the proteins was independently aligned with MUSCLE (Edgar, 2004), and the resulting alignments were then concatenated and used to construct the whole-genome Maximum-Likelihood phylogenetic tree. The generated newick tree was then visualized using MEGA X v10.0.5 (Kumar et al., 2018). For assays developed based on two or more genomes, the core genome of the target strains was searched for candidate chromosomal singletons against all other strains in the EDGAR project. For assays developed based on only one genome, the totality of the genome was used to search for singletons. An overview of the workflow is presented in Figure 1.

Since EDGAR uses a bidirectional best BLASTp hits approach for calculating singletons, gene duplication and paralogs are treated as singletons, if unidentical (Blom et al., 2016). The in silico specificity of each singleton was thus further verified using a BLASTn v2.7.1 analysis (Camacho et al., 2009) against a local database of the 20 genomes used for the genome-informed target identification including Xanthomonas spp. genomes sequenced at ZHAW (Table 1), and against the nucleotide nr/nt (Xanthomonadales, taxid:135614) NCBI collection (accessed February 26, 2020). To refine results, the singleton specificity was further checked using the whole-genome shotgun contigs (WGS) database (Xanthomonadales, taxid:135614) at NCBI (accessed on February 26, 2020), a crucial step in genomics-based assay development as the nucleotide and the WGS databases contain different types of genome sequence data.

To ensure that specific singletons are not erroneously discarded, the identity of the genomes with significant hits to the singletons of the various assays was verified by calculating average nucleotide identity (ANI) values using fastANI v1.1 (Jain et al., 2018) between the genome sequences. Singletons were considered specific and retained for primer design if BLASTn did not yield significant hits besides the respective target organism(s), if the genes were not located in the close vicinity (~10 kb on each side) of potentially functional transposases, if the genes were located on the chromosome (in complete genomes) or on contigs tentatively assigned as chromosomal, and if they were present in only one copy.

### LAMP Primer Design, Synthesis, and Selection

Primer sets (outer primers F3/B3, loop primers LoopF/LoopB and internal primers FIP/BIP) were designed using LAMP Designer v1.16 (Premier Biosoft, San Francisco, CA). Default reaction conditions and primer parameters were used. Depending on the number of specific singletons for the various assays (Supplementary Table 1), primer sets were designed for either the same or different singleton(s) (Supplementary Table 2). Primer sets without a secondary structure, with an increased stability at primer ends and an optimal distance between ends of F2 and B2 sequences, were selected for further analysis.
**In silico** specificity of the outer primers and their amplicons was analyzed using primer-BLAST and BLASTn, respectively, using databases as previously described (accessed February 28, 2020). Primer sets fulfilling both specificity criteria were selected for *in vitro* analysis. Based on LAMP primer design recommendations, “TTTT” linkers were added for the internal primers (Notomi et al., 2000). For each assay, four primer sets were synthesized (Microsynth AG, Balgach, Switzerland), except for the assay targeting *X. hortorum* pv. hederae, for which six primer sets were prepared.

To select the optimal LAMP primer set per target group, the selectivity of the four or six primer sets (Supplementary Table 2) was first tested on a sub-selection of eight representative strains within the *X. hortorum* – *X. hydrangeae* species complex using genomic DNA or boiled cells. For each assay, one primer set was then selected for further analysis based on two criteria: selective amplification of the genomic DNA of the target organism(s) within the *X. hortorum* – *X. hydrangeae* species complex, and, given fulfilment of first criterion, shortest amplification time. Finally, selectivity of assays was then confirmed on the normalized genomic DNA of strains within the reference group, and the assays were used to differentiate strains within the validation set. The sequences of the retained primer sets are reported in Supplementary Table 3. The locus tags, annotation and contig accession numbers of the retained singletons are described in Supplementary Table 4.

**General LAMP Experiment and Assay Conditions**

The LAMP reaction composition for a single sample is reported in Supplementary Table 5. In all experiments, ddH2O served as a template control (NTC). All measurements were carried on a Light Cycler 480 Real-Time machine (Roche Diagnostics, Rotkreuz, Switzerland) in white 96-well plates (Roche). The target temperature was 64°C, ramp rate was 2.2°C s⁻¹ and amplifications ran for 55 min. Samples with ambiguous amplifications (i.e., melting curve not in expected range, unexpected amplification) were repeated in triplicates. Melting curve analysis was conducted for 5 min from 64°C to 95°C at 0.01°C s⁻¹; melting temperatures of the assays are reported in Supplementary Table 3.

**Assay Metrics of LAMP Assays**

Assay metrics were based on the normalized genomic DNA of the reference strains and were calculated as follows (Blaser et al., 2018): the assay sensitivity (true-positive rate) was calculated using formula [1], while specificity (true-negative rate) was calculated using formula [2], and assay efficiency (correct assay results) with formula [3].
**Limits of Detection of DNA- and Crude Bacterial Cell-Based Assays**

The limits of detection (LoD) of the DNA-template based assays were tested with tenfold dilutions series from 1 ng µl⁻¹ to 1 fg µl⁻¹ of representative target strains, with and without spiking plant extract in experimental triplicates (Supplementary Table 6). In addition, the effect of non-host plant extract was tested on the performance of crude bacterial cell-based assays.

For the crude bacterial cell-template based assays, representing strains were grown overnight on NYGA at 28°C. Single colonies were picked and transferred in sterile 0.8% KCl, representative strains were grown overnight on NYGA at 28°C. Experimental duplicates were tested with tenfold dilutions series from 1 ng µl⁻¹ to 1 fg µl⁻¹ of representative target strains, with and without spiking plant extract in experimental triplicates.

Performance of Two Assays on Naturally Infected Hydrangea

In July 2021, the Plant Diagnostic Center at ILVO (Merelbeke, Belgium) received Hydrangea arborescens cv. ‘Annabelle’ plants exhibiting characteristic Xanthomonas-leaf spots from a retail nursery in Belgium. LAMP assays were done on the naturally infected leaves at ILVO (only with the X. hydrangeae assay) and at Zurich University of Applied Sciences (ZHAW, Wädenswil, Switzerland) (with the X. hydrangeae and species complex assays) by the same protocol. Brieﬂy, leaf-discs of 10 mm in diameter were cut out from the symptomatic leaves, vigorously bead-beaten and further processed as described above except that the limits of detection of these assays. In steel ball bearings tubes, 1 ml of bacterial suspension and 1 ml of lysis buffer (OptiGene Ltd) were pipetted to the previously normalized genomic DNA in experimental duplicates. Genomic copy equivalents were calculated using the DNA amount (in ng), a template of average size 5.5 Mb and average weight of a base-pair of 650 Daltons. A DNA of four genotypes was isolated from over 50 plant species, in different years (1942 to 2021) and 30 countries. Strains were obtained from various international culture collections and national diagnostic laboratories as indicated in Supplementary Table 9. The reference group included 24 strains from the X. hortorum – X. hydrangeae species complex, for which whole genomes were available (Table 1). These genomes were included in the development of the primer sets. The remaining strains were chosen to reﬂect the broad diversity within the Xanthomonas genus, in addition to six pseudomonads. The validation set comprised 60 strains for which no whole genome sequences were available. The partial gyrB gene of 38 strains within the validation set was sequenced (data not shown).

**Seven LAMP Assays Developed**

The recently published whole-genome phylogeny of the X. hortorum – X. hydrangeae species complex revealed three major clades (Figure 2): 1) clade A, encompassing X. hortorum cvs. gardneri, cynarae, vitians (clade A1) and pv. taraxaci (clade A2); 2) clade B, including X. hortorum pv. hederae (clade B1), pv. carotae (clade B2) and pv. pelargonii (clade B3); and 3) X. hydrangeae (clade C) (Dia et al., 2021). These clades served as a base for finding candidate and specific singletons (Supplementary Table 1), the latter being used as LAMP assay targets. Seven LAMP assays were thus developed, one for the overarching X. hortorum – X. hydrangeae species complex and six other assays, each targeting a separate clade within the species complex (Figure 2). More information on assay development (e.g., candidate and specific singletons, candidate primer sets and selected singletons) is provided as Supplementary Text 1.

**Assay Metrics of LAMP Assays**

The sensitivity, speciﬁcity, and performance of all seven assays was 100% (Table 3). Of the X. hortorum – X. hydrangeae strains within the reference group, the genomic DNA of 20 strains was selectively ampliﬁed with two assays: 1) the overarching species complex assay and, 2) one clade-speciﬁc assay. The genomic DNA of four Xanthomonas sp. strains, namely GBBC 1934, SB 3701, SB 3727 and Xc407, yielded an amplification with the assay targeting the species complex only (Supplementary Table 9). No ampliﬁcation was observed for the genomic DNA of the 38 strains not belonging to the X. hortorum – X. hydrangeae species complex (Table 2 and Supplementary Table 9).
Limits of Detection of Bacterial Genomic DNA- and Crude Bacterial Cell-Template Based Assays

All assays yielded a positive reaction with bacterial genomic DNA quantities down to at least 2.5 pg per reaction (Table 4), equivalent to 421 genome-copies (Supplementary Table 7). The consistent LoD varied between the assays. Without plant extract spiking, the consistent LoD was 2.5 pg per reaction for the assays targeting clade A1 and the species complex, and down to 250 fg per reaction for the other assays. Spiking of the reaction mix with plant extracts decreased the consistent LoD of assays targeting clades A1, A2, B3 and C tenfold, after spiking with plant extracts from lettuce, dandelion, pelargonium, or hydrangea. The consistent LoD of the assays targeting clades B1, B2 and the species complex were not affected by the addition of plant extracts.

When using boiled bacterial cells, all assays detected at least about 1,000 cells µl⁻¹ of LAMP reaction mix (equivalent to 1 × 10⁶ cells ml⁻¹) (Table 5 and Supplementary Table 8). Other assays, like the ones targeting clades A2 and B2, were also able to consistently detect 100 cells µl⁻¹ of LAMP reaction mix (equivalent to 1 × 10⁵ cells ml⁻¹). Representative amplifications by the assay targeting the species complex are shown in Figures 3A–D.

Practical Applications of the LAMP Assays

To examine the applicability of the assays, the seven assays were tested on a validation set of 60 unclassified strains or strains with an uncertain classification received as Xanthomonas spp. or X. hortorum from culture collections or diagnostic laboratories (Supplementary Table 9). The genomic DNA of 39 strains of this validation set yielded an amplification with the assay targeting the species complex, and with an assay targeting one of the six clades, assigning them to one of the defined clades. The genomic DNA of nine strains were only amplified with the assay targeting the complex, suggesting that these strains belong to the

### TABLE 2 | Reference strains used in this study and results of the LAMP assays based on their normalized genomic DNA. Selective amplification is marked with a sign “+”, and “-” denotes absence of amplification.

| Organism names as received | No. of strains | A1 | A2 | B1 | B2 | B3 | C | Xh-Xhydr species complex |
|-----------------------------|----------------|----|----|----|----|----|---|--------------------------|
| **X. hortorum – X. hydrangeae species complex** (n = 24) |                |    |    |    |    |    |   |                          |
| X. hortorum pv. vitians     | 3              | +  | -  | -  | -  | -  | - | +                        |
| X. hortorum pv. cynarae     | 2              | +  | -  | -  | -  | -  | - | +                        |
| X. hortorum pv. gardneri    | 3              | +  | -  | -  | -  | -  | - | +                        |
| “X. hortorum” pv. nigromaculans (= [X. campestris] pv. nigromaculans) | 1 | + | -  | -  | -  | -  | - | +                        |
| X. hortorum pv. taraxaci    | 1              | -  | +  | -  | -  | -  | - | +                        |
| X. hortorum pv. hederiae    | 3              | -  | -  | +  | -  | -  | - | +                        |
| X. hortorum pv. carotae     | 2              | -  | -  | -  | +  | -  | - | +                        |
| X. hortorum pv. pelargonii  | 1              | -  | -  | -  | -  | +  | - | +                        |
| X. hortorum [no pathovar]   | 4              | -  | -  | -  | -  | -  | + | +                        |
| X. hydrangeae               | 4              | -  | -  | -  | -  | -  | + | +                        |
| **Outgroup strains** (n = 38) |                |    |    |    |    |    |   |                          |
| X. atlatiae                | 2              | -  | -  | -  | -  | -  | - |                          |
| X. arboricola pv. juglandis | 2              | -  | -  | -  | -  | -  | - |                          |
| X. axonopodis              | 2              | -  | -  | -  | -  | -  | - |                          |
| X. bromi                   | 1              | -  | -  | -  | -  | -  | - |                          |
| X. campestris              | 2              | -  | -  | -  | -  | -  | - |                          |
| X. cassavae                | 2              | -  | -  | -  | -  | -  | - |                          |
| X. citri pv. citri         | 1              | -  | -  | -  | -  | -  | - |                          |
| X. codiae                  | 1              | -  | -  | -  | -  | -  | - |                          |
| X. cucurbitae              | 2              | -  | -  | -  | -  | -  | - |                          |
| X. floridensis             | 1              | -  | -  | -  | -  | -  | - |                          |
| X. hyacinthi               | 2              | -  | -  | -  | -  | -  | - |                          |
| X. malennis                | 1              | -  | -  | -  | -  | -  | - |                          |
| X. melonis                 | 2              | -  | -  | -  | -  | -  | - |                          |
| X. nasturtii               | 1              | -  | -  | -  | -  | -  | - |                          |
| X. perforans               | 1              | -  | -  | -  | -  | -  | - |                          |
| X. psi                     | 2              | -  | -  | -  | -  | -  | - |                          |
| X. populi                  | 1              | -  | -  | -  | -  | -  | - |                          |
| X. prunicola               | 1              | -  | -  | -  | -  | -  | - |                          |
| X. sacchari                | 1              | -  | -  | -  | -  | -  | - |                          |
| X. translucens pv. translucens | 1 | -  | -  | -  | -  | -  | - |                          |
| X. vasicola pv. holicola   | 1              | -  | -  | -  | -  | -  | - |                          |
| X. vescicatoria            | 2              | -  | -  | -  | -  | -  | - |                          |
| P. fluorescens             | 3              | -  | -  | -  | -  | -  | - |                          |
| P. syringae                | 3              | -  | -  | -  | -  | -  | - |                          |

*A list with all the strains tested is presented in Supplementary Table 9. **Clade A1: X. hortorum pv. cynarae, gardneri and vitians, clade A2: X. hortorum pv. taraxaci, clade B1: X. hortorum pv. hederiae, clade B2: X. hortorum pv. carotae, clade B3: X. hortorum pv. pelargonii, clade C: X. hydrangeae, Xh-Xhydr species complex: X. hortorum – X. hydrangeae species complex.
TABLE 3 | Specificity, sensitivity, and efficiency of the LAMP assays based on the genomic DNA of the 62 reference strains.

|                | A1a | A2 | B1   | B2   | B3   | C     | Xh-Xhydr species complex |
|----------------|-----|----|------|------|------|-------|--------------------------|
| Np             | 62  | 62 | 62   | 62   | 62   | 62    | 62                       |
| NTP            | 9   | 1  | 3    | 2    | 1    | 4     | 24                       |
| NTN            | 53  | 61 | 59   | 60   | 61   | 58    | 38                       |
| Nfp            | 0   | 0  | 0    | 0    | 0    | 0     | 0                        |
| Nfn            | 0   | 0  | 0    | 0    | 0    | 0     | 0                        |
| Assay sensitivity (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Assay specificity (%)  | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Test efficiency (%)   | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

aClade A1: X. hortorum pv. cynarae, gardneri and vitians, clade A2: X. hortorum pv. taraxaci, clade B1: X. hortorum pv. hederae, clade B2: X. hortorum pv. carotae, clade B3: X. hortorum pv. pelargonii, clade C: X. hydrangeae, Xh-Xhydr species complex. bWith spiking correspond here to the omission and addition of plant cell extracts in the steel ball-bearing tube used to prepare the LAMP DNA template, respectively. cNC: no change (i.e., same consistent and last detection limits).

FIGURE 2 | Whole-genome Maximum-Likelihood core phylogeny of the Xanthomonas hortorum–Xanthomonas hydrangeae species complex. The tree shows the various clades within the species complex (grey arrow): clades A1 (X. hortorum pv. gardneri, cynarae, vitians and X. hortorum pv. nigromaculans, grey square) and A2 (X. hortorum pv. taraxaci, blue circle), clades B1 (X. hortorum pv. hederae, green circle), B2 (X. hortorum pv. carotae, red circle) and B3 (X. hortorum pv. pelargonii, yellow circle), and clade C (X. hydrangeae, grey diamond) in addition to three outgroup strains. Accession numbers of the 20 genomes used in this study are noted in Table 1.

TABLE 4 | Summary of the consistent and last limits of detection (LoD) of genomic DNA-based assays. Genome-copy equivalents are reported between parentheses.

|                | A1a | A2 | B1 | B2 | B3 | C | Xh-Xhydr species complex |
|----------------|-----|----|----|----|----|---|--------------------------|
| Consistent LoD Without spiking | 2.5 pg (421) | 250 fg (421) | 250 fg (421) | 250 fg (421) | 250 fg (421) | 250 fg (421) | 2.5 pg (421) |
| Consistent LoD With spiking    | 25 pg (4210) | 25 fg (4210) | 250 fg (4210) | 250 fg (4210) | 250 fg (4210) | 250 fg (4210) | 2.5 pg (4210) |
| Last LoD Without spiking       | NC  | NC | NC | NC | NC | NC | 250 fg (421) |
| Last LoD With spiking          | 250 fg (421) | 250 fg (421) | 250 fg (421) | 250 fg (421) | 250 fg (421) | 250 fg (421) | 250 fg (421) |

aClade A1: X. hortorum pv. cynarae, gardneri and vitians, clade A2: X. hortorum pv. taraxaci, clade B1: X. hortorum pv. hederae, clade B2: X. hortorum pv. carotae, clade B3: X. hortorum pv. pelargonii, clade C: X. hydrangeae, Xh-Xhydr species complex. bWithout and with spiking correspond here to the omission and addition of plant cell extracts in the steel ball-bearing tube used to prepare the LAMP DNA template, respectively. cNC: no change (i.e., same consistent and last detection limits).
**TABLE 5** | Summary of the consistent and last limits of detection (LoD) of cell-based assays, reported in number of bacteria in 1 µl of LAMP reaction.

|                | A1* | A2 | B1 | B2 | B3 | C | Xh-Xhyd species complex |
|----------------|-----|----|----|----|----|---|------------------------|
| **Consistent LoD** | 1,000 | 100 | 1,000 | 100 | 1,000 | 1,000 | 1,000 |
| **Last LoD** | NC | 10 | NC | 100 | 1 | 100 |

*Clade A1: X. hortorum pv. cynarae, gardneri and vitians, clade A2: X. hortorum pv. taraxaci, clade B1: X. hortorum pv. hederae, clade B2: X. hortorum pv. carotae, clade B3: X. hortorum pv. pelargonii, clade C: X. hydrangeae, Xh-Xhyd species complex: X. hortorum – X. hydrangeae species complex. NC: no change (i.e., same consistent and last detection limits).

**DISCUSSION**

**Assay Development**

Assays were originally planned for each separate pathovar within clade A, in addition to an assay encompassing the totality of clade A. However, no suitable singletons were found between representative genomes of *X. hortorum* pv. gardneri, cynarae, vitians and taraxaci. Out of the four pathovars within that clade, suitable singletons were only found for *X. hortorum* pv. taraxaci. Thus, two assays were developed: one targeting specifically clade A1 containing *X. hortorum* pv. gardneri, vitians and cynarae and one targeting clade A2 specific for *X. hortorum* pv. taraxaci. This can be explained by the very high genetic relatedness between strains within the clade A1. The ANI values between strains of *X. hortorum* pv. cynarae, gardneri and vitians were above 98%, whereas ANI values between *X. hortorum* pv. taraxaci.
taraxaci (clade A2) and clade A1 strains were below 98% (Morinière et al., 2020). The threshold for finding singletons acceptable as genomic targets is thus related to the ANI values, and the higher the ANI value, the more challenging finding a suitable singleton.

**Limits of Detections**

Insufficient information is available concerning the LoD of the existing molecular diagnostic assays targeting single pathovars of *X. hortorum*. Out of 18 PCR-based and isothermal amplification studies present in the literature, only six reported LoD values (Dia et al., 2022) and these ranged from 2 pg to 200 fg for DNA experiments, whereas bacterial cell-based LoD values were between $5 \times 10^4$ CFU ml$^{-1}$ and $5 \times 10^6$ CFU ml$^{-1}$.

The consistent LoD of the DNA-template based assays developed in this study is comparable to LAMP and PCR assays previously published for detecting *X. hortorum* pv. gardneri (Stehliková et al., 2020) and *X. hortorum* pv. carotae (primer set 9B) (Meng et al., 2004). On the other hand, the LAMP assays developed in this study are more sensitive than the PCR/Multiplex-PCR assays targeting *X. hortorum* pv. gardneri for which LoD was estimated to be 50 pg µl$^{-1}$ (Araújo et al., 2012). The sensitivity of our assays, however, is lower than two previously published assays (conventional PCR and qPCR), targeting *X. hortorum* pv. carotae (primer set 3S) (Meng et al., 2004) and *X. hortorum* pv. pelargonii (Farahani and Taghavi, 2016). The greater sensitivity of qPCR when compared to LAMP has also been previously reported (Bühlmann et al., 2013). The assays in the current study on boiled bacterial cells had a consistent detection limit in the same order of magnitude as previously published studies (Araújo et al., 2012; Strayer et al., 2016; Strayer-Scherer et al., 2019).

**LAMP Assays to Identify *X. hortorum* – *X. hydrangeae* Strains**

All seven assays were highly sensitive and specific, irrespective of their taxon-level of detection. LAMP assays were able to assign reference strains, initially classified as *Xanthomonas* sp. based on partial sequence analysis of *gyrB*, to a level lower than the species complex (Table 2). The genomic DNA of those strains resulted in amplification with two assays (i.e., the assay targeting the whole complex and a single assay targeting one of the six clades). For example, the genomic DNA of strains GBBC 1967 and GBBC 950, both isolated from *Hedera helix*, was amplified by the assay targeting the whole complex and the one targeting *X. hortorum* pv. hederae (clade B1), assigning them to *X. hortorum* pv. hederae. This was further confirmed using whole-genome sequence data (data not shown).

Several strains from both the reference and validation set were accurately identified as belonging to the *X. hortorum* – *X. hydrangeae* species complex but their genomic DNA did not yield amplification by any of the six other assays targeting various clades. Based on LAMP results, this would suggest that they potentially belong to new clades within the *X. hortorum* – *X. hydrangeae* species complex. Some of these strains were isolated from plants that were not previously reported as host plants within *X. hortorum* (Dia et al., 2022). This suggests that several novel lineages may exist within the *X. hortorum* – *X. hydrangeae* species complex requiring further investigation. In case that novel lineages are confirmed, and depending on their relevance to plant health, the development of new assays targeting these novel lineages might be required.

Furthermore, results from the LAMP assays on the validation set also showed that several strains had been misassigned to the species complex by the diagnostic laboratories that provided the strains, as their genomic DNA did not yield amplification with any of the LAMP assays. More information regarding those strains is presented in Supplementary Text 2 and Supplementary Figure 1.

**Performance of Two Assays on Naturally Infected Hydrangea Plants**

The performance of diagnostic assays on field samples is a crucial metric of applicability as a diagnostic method. We previously had tested the LAMP assays to detect *X. hydrangeae* in artificially inoculated plants (Dia et al., 2021), using a 48-hour growth step. In this study, two LAMP assays were tested directly on extracts of naturally infected *Hydrangea* leaves. The time required from receiving the infected leaves to LAMP results was two hours, and it can be further lowered by using a shorter amplification time. No bacterial growth step was needed as leaf spot macerate was diluted and directly used as LAMP reaction template. The assays identified the causal agent of bacterial leaf spot of *Hydrangea* as a member of clade C (*X. hydrangeae*). The first assay targeting the whole *X. hortorum* – *X. hydrangeae* species complex provided additional certainty to the diagnosis results.

The LAMP assay targeting clade C was used at ILVO with the same protocol used at ZHAW except that it was performed on a portable machine. The same conclusion was drawn identifying *X. hydrangeae* as the causative agent of the bacterial leaf spot on hydrangea. This validation and transferability step is extremely important in diagnostics (EPPO, 2021), further attesting to the robustness of the developed assay. Additionally, the field-deployable capacity of the assays developed here was also demonstrated, a very valuable characteristic within plant pathogen diagnostics.

Even though the detection limits of some of the assays decreased when spiked with plant extracts, the results obtained from the two LAMP assays on naturally infected *Hydrangea* demonstrate the applicability of the assays on plants with an ongoing infection with *X. hortorum* – *X. hydrangeae* species complex strains. The performance of the assays in latent phases of infections and on other hosts remains to be assessed. This is of particular relevance as some *X. hortorum* members can survive on plant surfaces as epiphytes (Toussaint et al., 2012) and as the presence of non-pathogenic *Xanthomonas* species (Vauterin et al., 1996; Cesbron et al., 2015) cannot be excluded.

**Concluding Remarks**

The LAMP assays developed in this study can be used for multiple purposes, both *in silico* and in the laboratory. *In silico*, the specific singletons can be used to identify suspected strains within the *X. hortorum* – *X. hydrangeae* species complex from
their genome sequences and, potentially assign them to one of the defined clades without detailed analysis of the genomes. This is invaluable given the ever-changing pathogen taxonomy, not always quickly adopted in diagnostic or research laboratories, and the prevalent mischaracterization and misnaming of strains and genomes.

The LAMP assays can be used in field settings by using macerated and diluted symptomatic tissue as LAMP template. The LAMP assays can also be used in a laboratory setting, for example, to (re)assign strains within bacterial collections or to identify *X. hortorum* – *X. hydrangeae* strains when tested on extracted genomic DNA or crude boiled bacterial cells. In this study, we have shown that the LAMP assays were able to assign previously unassigned strains to clades within the *X. hortorum* – *X. hydrangeae* species complex. Some strains could be assigned to the species complex but not to a lower taxonomical level. These strains could represent novel groups within the complex and require further phylogenetic analysis. The combination of various taxon detection levels (i.e., species to pathovar level) provided a higher degree of discriminative power, in addition to minimal sample preparation and post-amplification handling (e.g., no gel electrophoresis step), higher tolerance to inhibitors present in crude plant samples and with field-deployable capacities.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

ND, TS, and JP conceptualized the study and analyzed the data. ND and JP created the methodology, and curated the data with the assistance from JB. ND, BC, TS, and JP were involved in the investigations. JB and JP helped with software. ND, BC, TS, and JP were involved in the validation. ND, TS, and JP contributed to the data visualization. TS and JP contributed to project administration and supervision. ND, TS, and JP prepared the original draft with assistance from BC and JB for review and editing. All the authors revised the final version of the manuscript, while JP acted as the corresponding author. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2022.898778/full#supplementary-material

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