Xanthomonas hydrangeae sp. nov., a novel plant pathogen isolated from *Hydrangea arborescens*

Nay C. Dia[1,2], Johan Van Vaerenbergh[3], Cinzia Van Malderghem[3], Jochen Blom[4], Theo H.M. Smits[1], Bart Cottyn[3], Joël F. Pothier[1]

[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, Zurich, Switzerland.

[3] Plant Sciences Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium.

[4] Bioinformatics and Systems Biology, Justus-Liebig University Giessen, Giessen, Germany

*Corresponding author: Joël F. Pothier joel.pothier@zhaw.ch

Keywords: overall genome-relatedness indices; *Xanthomonas*; diagnostics

Repositories: The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* sequences of strain type LMG 31884^T are OU231258 and MW223005, respectively. The EMBL accession numbers for genome sequences of LMG 31884^T, LMG 31887, LMG 31885 and LMG 31886 are GCA_905142475, GCA_905142485, GCA_905142495 and GCA_905142465, respectively.

ABSTRACT

This paper describes a novel species isolated in 2011 and 2012 from nursery-grown *Hydrangea arborescens* cultivars in Flanders, Belgium. After four days at 28°C, the strains yielded yellow, round, convex and mucoid colonies. Pathogenicity of the strains was confirmed on its isolation host, as well as on *Hydrangea quercifolia*. Analysis using MALDI-TOF MS identified the *Hydrangea* strains as belonging to the genus *Xanthomonas* but excluded them from the species *Xanthomonas hortorum*. A phylogenetic tree based on *gyrB* confirmed the close relation to *X. hortorum*. Three fatty acids were dominant in the *Hydrangea* isolates: anteiso-$C_{15:0}$, iso-$C_{15:0}$ and summed feature 3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$). Unlike *X. hortorum* pathovars, the *Hydrangea* strains were unable to grow in presence of lithium chloride and could only weakly utilize D-fructose-6-PO_4 and glucuronamide. Phylogenetic characterization based on multilocus sequence analysis and phylogenomic characterization revealed that the strains are close to, yet distinct from, *X. hortorum*. The

genome sequences of the strains had an Average Nucleotide Identity (ANI) ranging from 94.35 to 95.19%, and *in silico* DNA-DNA hybridization (*is*DDH) values ranging from 55.70 to 59.40% to genomes of the *X. hortorum* pathovars. A genomics-based loop-mediated isothermal amplification (LAMP) assay was developed which was specific to the *Hydrangea* strains for its early detection. A novel species, *Xanthomonas hydrangeae* sp. nov., is proposed with strain LMG 31884^T (=CCOS 1956^T) as the type strain.

INTRODUCTION

The genus *Xanthomonas*, first described by Dowson in 1939, consists of Gram-negative plant pathogenic bacteria that are rod-shaped with a single flagellum and which produce yellow-pigmented colonies [1]. The genus currently comprises 29 validly published species (https://lpsn.dsmz.de/genus/xanthomonas; accessed in September 2021), causing diseases in more than 400 plant hosts, including stone fruits, citrus, ornamental plants, and grasses such as rice and wheat [2]. The original classification of the genus was based on a phenetic approach focusing on one phenotypic trait, namely host specificity. However, many *Xanthomonas* species were demonstrated to be heterogeneous based on a number of techniques, e.g., protein electrophoresis [3], restriction fragment length polymorphism (RFLP) on genomic DNA [4] and DNA-DNA hybridization [3,5]. This led to an extensive revision of the *Xanthomonas* taxonomy, resulting in 20 nomenspecies based on DNA-DNA hybridization by Vauterin *et al.* [6]. Parkinson *et al.* [7,8] later demonstrated the genetic relatedness between the different *Xanthomonas* spp. using partial *gyrB* gene sequences. In these studies, some strains were clustered into different species-level clades (slc), the members of which share a common ancestor before final speciation [8]. One example of a species-level clade is the *Xanthomonas hortorum* slc. The relatedness of members of the *X. hortorum* slc was confirmed by two multilocus sequence analysis (MLSA) studies using partial sequences of four (*dnaK*, *fyuA*, *gyrB* and *rpoD*) [9] or even six (*atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, and *rpoD*) [10] housekeeping genes.

Recently, the taxonomy of the *X. hortorum* slc has further changed: *Xanthomonas cynarae* and *Xanthomonas gardneri* were initially reclassified as *X. cynarae* pv. cynarae and *X. cynarae* pv. gardneri [11]. The classification of the two pathovars was further modified to *X. hortorum* pv. cynarae and pv. gardneri, respectively [12]. Thus, the most recent taxonomy of *X. hortorum* includes one species (*X. hortorum*) with seven pathovars, namely *X. hortorum* pv. carotae, *X. hortorum* pv. hederae, *X. hortorum* pv. pelargonii, *X. hortorum* pv. taraxaci, *X. hortorum* pv. vitians, *X. hortorum* pv. cynarae (syn. *X. cynarae*; *X. cynarae* pv. cynarae) and *X. hortorum* pv. gardneri (syn. *X. gardneri*; *X. cynarae* pv. gardneri). It has also been

proposed that [*Xanthomonas campestris*] pv. nigromaculans should be renamed to *X. hortorum* pv. nigromaculans [8]. However, since it has not yet been formally described as such, it will not be officially considered as a pathovar of *X. hortorum* but will be included in analyses whenever possible.

The seven pathovars of *X. hortorum* cause diseases on a wide range of primary host plants, including crops, ornamentals, and wild plants. *X. hortorum* pv. cynarae (syn. *X. cynarae*) causes water-soaked spots on artichoke bracts [11,13]. *X. hortorum* pv. gardneri (syn. *X. gardneri*) is one of the three *Xanthomonas* species that causes bacterial spots on tomatoes and peppers [11,14]. *X. hortorum* pv. gardneri has been classified as an organism recommended for regulation by the European Plant Protection Organization (EPPO) since 2015, and as a regulated, non-quarantine pest (RNQP) by the EU since 2020 [15]. *X. hortorum* pvs. carotae, pelargonii and taraxaci are the causal agents of bacterial blight, characterized by angular leaf spots on carrots, geraniums and dandelions, respectively [12,16–18]. *X. hortorum* pvs. hederae and vitians cause bacterial spots on ivy and lettuce, respectively [12,19,20].

In this study, we report on four strains isolated in Belgium between 2011-2012 from container-grown *Hydrangea arborescens* plants [21]. The objective of this study was to characterize these strains through a polyphasic approach combining phylogenetic characterizations based on 16S rRNA gene sequencing, a MLSA of seven housekeeping genes (*atpD, dnaK, efp, glnA, gyrB, lepA* and *rpoD*) and a phylogenomic analysis. Furthermore, pathogenicity tests, Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometer (MALDI-TOF MS) peptide mass fingerprinting, phenotypic profiling and a fatty acid methyl esters (FAME) analysis were carried out. Overall-genome relatedness indices (i.e. Average Nucleotide Identity ANI and *in silico* DNA-DNA-Hybridization *is*DDH) were calculated. Moreover, we propose a comparative genomics-based, loop-mediated isothermal amplification (LAMP) diagnostics method for the rapid and selective identification and detection of this new species.

ORIGIN AND ISOLATION

Hydrangea arborescens plants with leaf spot symptoms were received at ILVO's Plant Diagnostic Center (PDC) from three Belgian ornamental nurseries in 2011 and 2012 [21]. The symptomatic plants were from four different cultivars (i.e., cvs. Bella Anna, Annabelle, Invincibelle and Incrediball). Bacteria were isolated from leaf spots at ILVO-PDC. Four representative isolates were selected for further analysis: LMG 31884^T (= CCOS 1956^T,

GBBC 2123^{T}), LMG 31885 (= CCOS 1954, GBBC 2128), LMG 31886 (= CCOS 1957, GBBC 2199) and LMG 31887 (= CCOS 1955, GBBC 2202).

LMG 31884^{T} was isolated in 2011 and originated from a Belgian nursery which had obtained rooted cuttings from its subsidiary company in Ethiopia. The mother plants to produce cuttings at the Ethiopian subsidiary were purchased in the USA. LMG 31887 was isolated in 2012 and was obtained from a second nursery that had received planting stock from the first nursery. LMG 31885 and LMG 31886 were isolated in 2011 and 2012, respectively, and originated from a third nursery which had imported the planting stock from the Netherlands. Tracking and tracing of the origin of the plants suggests that there was more than one introduction of the pathogen.

GROWTH AND INITIAL CHARACTERIZATION

The bacterial isolates were first grown on *Pseudomonas* agar F medium (Life Technologies Europe BV, Merelbeke, Belgium) for four days at 28°C. They were subsequently grown 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) for two days at 28°C. On both media, yellow, round, convex and mucoid colonies were observed.

Isolates were stored in a 1:1 (*v:v*) solution of nutrient-yeast glycerol broth (NYGB) and glycerol at -80°C. Strains were routinely plated on NYGA and incubated at 28°C for 48 h. The strains were deposited at the BCCM/LMG (Belgian Co-ordinated Collections of Micro-organisms / Laboratory of Microbiology, Ghent University, Belgium) and the CCOS (Culture Collection of Switzerland, Wädenswil, Switzerland). Information on their host, country and year of isolation is given in Table 1.

The partial *gyrB* gene of the isolates was amplified using *gyrB* primers XgyrPCR2F and X.gyrrsp1, as described by Parkinson *et al.* [7], and purified PCR products were sequenced (Genewiz, Leipzig, Germany). Based on the *gyrB* phylogenetic tree, the isolates likely represented a new species, with *X. hortorum* as the closest relative [21].

PATHOGENICITY TESTS

The pathogenicity of two isolates (LMG 31884^{T}, and LMG 31886) was tested by pressurized spray inoculation on the leaves of *H. arborescens* cv. Annabelle, *H. quercifolia*, *Hydrangea macrophylla* cv. Zorro and *Hydrangea paniculata* cv. Pinky Winky, in addition to *Hedera* cv. Hibernica (Atlantic ivy), *Pelargonium zonale*, *Taraxacum kok-saghyz* (Russian dandelion) and *Daucus carota* cv. Nerac (carrot). Fresh, pure bacterial cultures of the bacterial strains were grown for 48 h at 28°C on NYGA-Sucrose media, and were used to prepare the

inoculum into a mixture of 10 mM Phosphate-Buffer Saline (PBS) - 0.02% Tween. The inoculum was diluted to 10^6 cfu/ml based on OD_{600} measurement. The abaxial side of the leaves were pressurized-sprayed with either the inoculum (three plants/inoculum) or the PBS-Tween solution, serving as a control (one plant/inoculum). The plants were placed in plastic boxes in a greenhouse; the boxes were covered with a plastic sheet for two days to maintain high humidity. Plants were checked daily for symptoms and irrigated when needed.

Regular leaf spots developed only on *H. arborescens* at 28 days post-inoculation (Fig. 1a). Few spots developed on *H. quercifolia* (Fig. 1b). No spots developed on *H. macrophylla* and on *H. paniculata*, while symptoms were not detected on the tested host plants for *X. hortorum* pathovars (Atlantic ivy, *Pelargonium zonale*, Russian dandelion, or carrot; data not shown). Furthermore, *X. hortorum* pv. carotae strain CFBP 7900 did not develop spots on *H. arborescens* (data not shown). The results show that the homologous plant host, *H. arborescens*, is the most susceptible of the *Hydrangea* species tested, but only for the *Hydrangea* strains. To fulfil Koch's postulates, selected spots were macerated in PBS and then plated in serial dilutions. Partial sequences of *gyrB* or *rpoD* from yellow, mucoid characteristic *Xanthomonas* colonies were determined and the sprayed strains were confirmed to be the causal agents of the observed symptoms (data not shown).

MALDI-TOF MS ANALYSIS

The four *Hydrangea* strains were analyzed through a Bruker MALDI-TOF MS Biotyper (Bruker Daltonics, Billerica, MA) identification system. Strains were grown in tryptic soy broth at 28°C with shaking at 220 rpm for 24 h before centrifuging 1 ml at 11,000 *g* for 2 min and inactivating the pellet by resuspending in 250 µl 70% ethanol for a minimum of 1 min. The inactivated cells were then pelleted as described above. The pellet was dried for a minimum of 5 min at room temperature, resuspended in 25 µl of 70% formic acid and diluted *v*/*v* with acetonitrile. After another centrifugation step as above, 1 µl supernatant was spotted on a steel standard target and air-dried completely. The spot was then overlayed with 2 µl of a 40 mg ml^{-1} alpha-cyano-4-hydroxy-cinnamic acid matrix and air-dried before analysis. Log(score) values were calculated against the Bruker database v..1 (6,903 strains, including 36 *Xanthomonas* species).

High confidence identification scores (2.00 to 3.00; secure species) were only obtained for *X. hortorum* pv. hederae CFBP 4925^T, reaching between 2.01 and 2.27 with various strains of *X. hortorum* pv. pelargonii and *X. hortorum* pv. cynarae (Table S1). The four *Hydrangea* strains yielded lower scores (scores ≤ 2.07; Table S1), with the majority of scores between 1.70 and 1.99 (low confidence identification; secure genus). The identification based on Bruker Biotyper thus shows that the strains undeniably belong to the genus *Xanthomonas*,

with *X. hortorum* as the closest relative, but there is substantial doubt that the *Hydrangea* strains belong to *X. hortorum* as the log(score) values were too low to support a solid inclusion in the species.

FATTY ACID METHYL ESTER ANALYSIS

The whole-cell fatty acid methyl ester (FAME) compositions of the strains were analyzed using a 6890 gas chromatograph (Agilent Technologies, Palo Alto, California, USA). Strains LMG 31884^{T} and LMG 31886, in addition to *X. hortorum* pv. carotae CFBP 7900, pv. pelargonii CFBP 2533^{PT}, pv. hederae CFBP 4925^{T}, pv. taraxaci NCPPB 940^{PT} and pv. vitians LMG 838^{neoPT}, were grown on NYGA plates at 28°C for 48 h before FAME analysis. The fatty acid methyl esters were then extracted and identified following the protocol of Sherlock Microbial Identification System (MIDI, Newark, Delaware, USA). Peaks were identified using the TSBA6 method and the Sherlock library database v.6.1.0.

Three fatty acids constituted between 56% to 65% of the total fatty acid composition (Table S2): anteiso-C15:0, iso-C15:0 and summed feature 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*). The three fatty acids characteristic of the *Xanthomonas* genus, iso-$C_{11:0}$, iso-$C_{11:0}$ 3-OH and iso-$C_{13:0}$ 3-OH [6], were present in all tested strains. There were three minor compositional differences between strains LMG 31884^{T} and LMG 31886. All strains contained eleven fatty acids in amounts larger than 1%. There were no fatty acid composition patterns differentiating the strains LMG 31884^{T} and LMG 31886 from *X. hortorum* pathovars.

PHENOTYPIC CHARACTERIZATION

Utilization of carbon sources by strains LMG 31884^{T} and LMG 31885 and their resistance to certain chemical compounds were characterized using GEN III MicroPlates (Biolog, Hayward, United States). Experiments were carried out in triplicates. Strains were subcultured from glycerol stock, grown on NYGA medium for 48 h at 28°C and then grown overnight on Biolog Dehydrated Growth agar at 28°C. Fresh colonies were transferred into Biolog Inoculating Fluid A using cotton swabs. The inoculum density was adjusted to a transmittance of 95-98%. A volume of 100 µl of inoculum was pipetted into wells of the MicroPlate, which was incubated at 28°C. The MicroPlates were measured using a MicroStation 2 Reader (Biolog) after 72 hours of incubation. The utilization data was first compared to the water no-substrate control (NSC); results were considered positive if 160% higher than the NSC and negative if 130% or less than the NSC values, to subtract the no-substrate baseline. Results falling between those two values were qualified as weak [22].

Differential phenotypes are listed in Table 2, while the full phenotypic profiles are reported in a supplementary table (Table S3). Across all triplicates, both LMG 31884^{T} and LMG 31885

unequivocally utilized 33 substrates, weakly utilized six substrates, and could not utilize five substrates (Table S3). For the rest of the substrates, the results were inconclusive as they were inconsistent across the replicates. Strains LMG 31884^{T} and LMG 31885 can be distinguished from all *X. hortorum* pathovars by their inability to grow in the presence of lithium chloride and their ability to weakly utilize D-fructose-6-PO_4 and glucuronamide (Table 2).

GENOME FEATURES

The DNA extraction, genome sequencing and subsequent assembly and annotation were carried out as previously described [23]. The genome sequences of strains LMG 31884^{T}, LMG 31887, LMG 31885 and LMG 31886 were deposited in EMBL, and their accession numbers are ERZ1690675, ERZ1690678, ERZ1690676 and ERZ1690677, respectively (Table 3). The genome of LMG 31884^{T} is complete and consists of a circular chromosome and four circular plasmids pLMG31884_p76, pLMG31884_p73, pLMG31884_p46 and pLMG31884_p23 (Table 3). A total of 4,703 CDS were annotated for the LMG 31884^{T} genome. Its G+C content of 63.6 mol% is within the typical range of *Xanthomonas* genomes. The genomes of the three other *Hydrangea* strains are complete, except one gap in strain LMG 31885 that could not be resolved, yielding the genome of this strain to be not circular. Strains LMG 31887 and LMG 31885 harbor two plasmids each, while strain LMG 31886 has only one plasmid.

The four *Hydrangea* strains share a core genome of 4,122 CDS and each strain has specific singletons (average of 53 singletons, ranging between 1 and 112 singletons). Strain LMG 31884^{T} and strains *X. hortorum* pv. vitians LMG 938^{neoPT}, *X. hortorum* pv. cynarae CFBP 4188^{PT}, *X. hortorum* pv. gardneri ATCC 19865^{PT}, *X. hortorum* pv. hederae CFBP 4925^{T}, *X. hortorum* pv. carotae CFBP 7900, *X. hortorum* pv. taraxaci NCPPB 940^{PT}, *X. hortorum* pv. pelargonii CFBP 2533^{PT} and [*X. campestris*] pv. nigromaculans NCPPB 1935 share a core genome of 3,122 CDS.

The partial 16S rRNA gene sequences of the type strains of *Xanthomonas* species, in addition to one *Xyllela fastidiosa* strain, ATCC 35879^{T}, serving as an outgroup, were retrieved from the List of Prokaryotic names with standing in nomenclature (LPSN; access date January 2021). The partial 16S rRNA gene sequences of the four *Hydrangea* strains and 13 *X. hortorum* strains were retrieved from their genomes. The 16S rRNA gene sequences were aligned using MEGA X v.10.1.7 [24] and a Maximum-Likelihood phylogenetic tree was constructed (1,227 positions, 1,000 bootstraps). The 16S rRNA gene-based phylogeny (Fig. 2) displays two main clades within the genus *Xanthomonas*, with one of them including all *X. hortorum* slc strains.

The 16S rRNA gene sequences of strains LMG 31884^T, LMG 31887, LMG 31885 and LMG 31886 differed by one nucleotide at position 364: G for strains LMG 31884^T and LMG 31887, and A for strains LMG 31886 and LMG 31885. The 16S rRNA gene sequences of the *Hydrangea* strains, the *X. hortorum* strains and *Xanthomonas* type species *X. campestris* pv. campestris strain ATCC 33913^T differed by three nucleotides at positions 342, 364 and 894, corresponding to a variation of less than 0.3%. Furthermore, there were only 52 variable nucleotides (4.24% variation) over the full 16S rRNA gene sequences of the *Xanthomonas* strains used in this phylogenetic study. The 16S rRNA gene is not variable enough within the genus *Xanthomonas* to be sufficiently discriminative between species [25].

MLSA with multiple housekeeping genes provided higher discriminative phylogeny resolution within the genus [26]. Twenty strains, namely the four strains from *Hydrangea,* thirteen strains of *X. hortorum* and three outgroup strains (*X. arboricola* pv. juglandis CFBP 2528^T, *X. populi* CFBP 1817^T and *X. campestris* pv. campestris ATCC 33913^T), were used to conduct MLSA based on the almost-complete concatenated sequences of seven housekeeping genes *atpD* (1,407 bp), *dnaK* (1,926 bp), *efp* (567 bp), *glnA* (1,404 bp), *gyrB* (2,442 bp), *lepA* (1,791 bp) and *rpoD* (1,878 bp). Sequences of the seven housekeeping genes of *X. hortorum* pv. carotae M081 (CFBP 7900; Table S4) were used as a query to retrieve closest orthologs (Table S5) from the genomes using tBLASTn (50% coverage, 60% hsp identity). A maximum likelihood phylogenetic tree (Fig. 3) was constructed after alignment in MEGA X [24] using the General Time Reversible model and a Gamma distribution with invariant sites [27]. Phylogeny was tested using 1,000 bootstrap replications, and bootstrap values below 50% were removed. The alignment of almost-complete concatenated sequences was used to trim the loci to the partial gene sequences previously described [9]. A similar topology was obtained (Fig. S1). Trimming settings and housekeeping gene lengths are reported in Table S6.

The four *Hydrangea* strains form a separate cluster within the *X. hortorum* slc; the closest *X. hortorum* strain is *X. hortorum* pv. pelargonii CFBP 2533^{PT}, while its closest non-slc relative is *X. populi* CFBP 1817^T. This is again indicative that the four *Hydrangea* strains may represent a separate taxon.

WHOLE-GENOME PHYLOGENY

Genome sequences of the aforementioned 20 strains were uploaded to the Type (Strain) Genome Server (available at https://tygs.dsmz.de) for a whole genome-based taxonomic analysis [28]. Intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.4 including SPR postprocessing [29]. Branch support

was inferred from 100 pseudo-bootstrap replicates each. The tree (Fig. 4) was rooted at the midpoint [30] and visualized with PhyD3 v.1.3 [31].

Whole-genome phylogeny reveals that strains of the *X. hortorum* pathovars form two major subclusters: subcluster A, encompassing *X. hortorum* pv. gardneri, pv. cynarae, pv. vitians and pv. taraxaci; and subcluster B, including *X. hortorum* pv. pelargonii, pv. hederae and pv. carotae. *X. hortorum* subcluster B is the closest phylogenetic relative of the *Hydrangea* strains (Fig. 4). Thus, both the MLSA-based and whole-genome phylogenies revealed three clusters within the *X. hortorum* slc: *X. hortorum* subclusters A and B and the cluster formed by the four *Hydrangea* strains.

OVERALL GENOME-RELATEDNESS INDICES

To investigate the degree of relatedness of these three clusters, overall genome-relatedness indices ANI and *is*DDH values were calculated for the same 20 genomes used in the previous analyses (Fig. 5). ANI values were calculated using fastANI, an alignment-free whole-genome ANI method [32], and *is*DDH was calculated using the genome BLAST distance phylogeny approach (GBDP) [33].

Within the cluster formed by the *Hydrangea* strains, fastANI and *is*DDH values ranged between 99.23 and 99.99% ANI, respectively, and 92.50 and 99.70% *is*DDH, respectively, clearly exceeding the 95-96% ANI and 70% *is*DDH thresholds for species delineation [32,33].

X. hortorum subcluster B is the closest relative to the genomes of the four strains from *Hydrangea*. Between those two entities, fastANI and *is*DDH values ranged between 94.84 and 95.19% ANI, respectively, and between 58.20 and 59.40% *is*DDH, respectively (Fig. 5). The *is*DDH values were well below the thresholds for species delineation, and even below the *is*DDH transition zone of 60-70%. Some of the fastANI values were above 95%, with the highest being 95.19%. The fastANI and *is*DDH values for *X. hortorum* subcluster A, in comparison to the *Hydrangea* strains, ranged between 94.35% and 94.99% ANI, and between 55.70% and 58.60% *is*DDH (Fig. 5). Both ANI and *is*DDH ranges fall below the species threshold values of 95% and 70%, respectively, and are also below the transition thresholds.

ANI values between 95-96% and 65-75% *is*DDH [34] are considered as falling in the “grey-zone” or “transition-zone”. In this study, fastANI values between the *Hydrangea* strains and two pathovars of *X. hortorum*, pvs. carotae and hederae fell into this “transition-zone”, whereas all *is*DDH values between the *Hydrangea* strains and *X. hortorum* were below 60%. Since our genome dataset includes genomes with varying degrees of completeness, fastANI

is the preferred method as it is more robust than alignment-based methods like ANIm (ANI calculation based on MUMmer) or ANIb (ANI calculation based on BLAST+ alignments) in the presence of draft genomes [32].

Nevertheless, to further investigate the relatedness between the *Hydrangea* strains and its closest phylogenetic relative *X. hortorum* subcluster B, alignment based ANIm and ANIb values between those two clusters (Table S7) were calculated using JSpeciesWS v.3.8.2 [35], as these two methods are still very commonly used for *Xanthomonas*.

ANIm values between the *Hydrangea* strains and the three *X. hortorum* pathovars in subcluster B ranged between 94.87 and 95.15% ANIm, with 78.75 to 84.95% of the sequences aligned. ANIb values between those same groups ranged from 94.16 to 94.5% ANIb, with 82.6 to 75.9% of sequences aligned. ANIm values are in accordance with the alignment-free fastANI values; indeed, ANIm values between *X. hydrangeae* and *X. hortorum* pvs. carotae and hederae, similarly to those calculated using fastANI, fall in the transition-zone, while ANIb values are below 95%.

Between the two methods that rely on sequence alignment, ANIm is the most robust as evidenced by the increased sequence alignment percentage when compared to ANIb. This is consistent with published results, reporting that ANIm yields more accurate results than ANIb especially when genomes compared have ANI values higher than 90% [34].

When ANI and/or *is*DDH values between strains fall in the transition zone, the decision to consider the strains as either the same or a distinct species is based on other genomic, phylogenetic and/or phenotypic analyses. Thus, even though some ANI values between the *Hydrangea* strains and *X. hortorum* were at the lower range of the “transition zone”, the remaining analyses, such as *is*DDH values below the “transition zone” and phenotypic traits discriminating between *X. hydrangeae* and all the *X. hortorum* pathovars, suggest the *Hydrangea* strains represent members of a novel species. By contrast, within *X. hortorum* pathovars, ANI and *is*DDH values do not warrant their separation into distinct species since other genomic, phenotypic, and phylogenetic analyses strongly point to the pathovars belonging to *X. hortorum* [12].

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION-BASED DIAGNOSTICS ASSAY

For taxonomy, two aspects are important: definition of a species as given above, but also diagnostics, e.g., for the identification of this organism in laboratory settings or more preferably in the field. We developed a highly species-specific loop-mediated isothermal amplification (LAMP) assay that can distinguish the *Hydrangea* strains from all the pathovars of its closest relative *X. hortorum* and around 70 other *Xanthomonas* and non-*Xanthomonas*

strains. This genomics-informed LAMP assay serves as an alternative to the phenotypic characterization.

In silico *assay development*

The assay was developed using a singleton coding sequence (CDS) approach on the EDGAR 3.0 platform [36]. Four CDS were retained for further analysis (Table S8). LAMP primer sets (F3/B3, LoopF/LoopB and FIP/BIP) were designed for each of the four specific CDS using LAMP Designer v.1.16 [37]. After a primer-BLAST analysis, one primer set, *Xhyd*-B (Table 4), was selected for the *in vitro* testing of the assay. Primer set *Xhyd*-B targets a 150 bp region of a 1,116 bp chromosomal CDS (annotated as *hypothetical protein*).

Performance of LAMP assay in a laboratory setting

Amplification was carried out on a LightCycler480 (Roche, Basel, Switzerland). Primer set *Xhyd*-B was first tested on boiled cells of the *Hydrangea* strains and had a mean amplification time of 9.62 min. This primer set was further tested on the normalized genomic DNA (1 ng/µl) of 88 strains (Table S9), namely ten strains isolated from *Hydrangea* plants including the four strains characterized in this study, 39 *X. hortorum* slc strains and 39 strains representing 30 non-*X. hortorum* slc species. Only the target strains were amplified within 20 min (Table S9), thereby confirming the presence of six further strains closely related to the *Hydrangea* ones. Assay performance metrics were calculated as reported by Blaser *et al.* [38]. The specificity, sensitivity and efficiency of the *Xhyd*-B LAMP assay were 100%, 97.3% and 97.7%, respectively.

Performance of LAMP assay on blind samples

The performance of the LAMP assay was tested on thirteen blind spot samples. Briefly, *Hydrangea* plants were inoculated at ILVO (Merelbeke, Belgium) as previously described [21]. Upon being received at ZHAW, each spot specimen (ca. 1 cm^2) was diagonally cut, and one part of the spot was transferred into a 15 ml tube with 5 ml of NYGB and incubated for 48 h with shaking at 220 rpm at 28°C. Around 1-2 ml of suspension was then transferred into a 1.5 ml tube in duplicates and centrifuged at 20,000 *g* for 5 min. The pellets were resuspended in 200 µl of ddH_2O. The suspensions were boiled at 95°C and directly used as DNA template for the LAMP reaction as previously described. For each sample, two suspensions were prepared, and measurements were taken in triplicates. A sample was only considered positive if all six instances were positive (e.g., amplification within 15-20 min). Samples with inconclusive results (e.g., inconsistent results between the biological duplicates or the experimental triplicates) were measured a second time.

Results of the LAMP assay were compared with isolation assays performed at ILVO from identical spot symptoms on leaves from the same plant sent to ZHAW. The re-isolated strains were confirmed to be identical to the spray-inoculated strains based on their *gyrB* sequences. The LAMP assay correctly assessed 11 out of the 13 samples, an accuracy based on blind samples of around 85% (data not shown). One sample was a false negative and the other one a false positive.

Evidence for assigning the *Hydrangea* strains to a new species

We have provided evidence which shows that the four *Hydrangea* strains represent members of a novel species, phylogenetically close, yet distinct to *X. hortorum*. Of the tested plants, *H. arborescens* is the only host for the strains. Outcomes of the Bruker MALDI-TOF MS analysis showed that the *Hydrangea* strains should be included in the genus *Xanthomonas*, but not within the species *X. hortorum*. This was confirmed by ANI and *is*DDH values for the *Hydrangea* strains cluster, which were well below the thresholds for species delineation. Although only few phenotypic traits allow a discrimination from *X. hortorum*, the phylogenetic and phylogenomic analyses showed that the *Hydrangea* strains consistently form a distinct, distant cluster to the species *X. hortorum*. For this reason, we propose the name *Xanthomonas hydrangeae* sp. nov. as a new member within the genus *Xanthomonas* to accommodate the *Hydrangea* strains, with strain LMG 31884^{T} (CCOS 1956^{T}=GBBC 2123^{T}) as its type strain. Along with the four strains characterized in this study, six further strains were identified by the LAMP assay that is highly specific for this novel species.

DESCRIPTION OF *XANTHOMONAS HYDRANGEAE* SP. NOV.

Xanthomonas hydrangeae (hy.dran.ge'ae. N.L. gen. n. *hydrangeae* of *Hydrangea*).

Cells are Gram-negative, motile, non-sporulating straight rods, and form colonies that are round, convex and mucoid when grown on Pseudomonas agar F for 4 days or nutrient-yeast glycerol agar for 2 days at 28°C. Produce yellow pigment. Catalase- and oxidase-negative. The optimal temperature for growth is between 25°C and 28°C. Grow at pH 7.0 and 6.0 but not at pH 5.0, and in the presence of NaCl concentrations up to 1%. Weak growth was observed at NaCl concentrations of 4%, while no growth was obtained at 8%. Grow in the presence of 1% sodium lactate, tetrazolium violet or tetrazolium blue, and in presence of antibiotics rifamycin or lincomycin, but not in presence of lithium chloride, sodium bromate, and potassium tellurite. Utilize D-trehalose, D-cellobiose, sucrose, D-melibiose, N-acetyl-D-

glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, glycerol, gelatin, glycyl-L-proline, L-alanine, L-glutamic acid, L-serine, pectin, methyl pyruvate, L-lactic acid, citric acid, α-keto-glutaric acid, L-malic acid, bromo-succinic acid, Tween 40, propionic acid and acetic acid as carbon or nitrogen sources. Do not utilize D-maltose, D-turanose, stachyose, D-raffinose, β-methyl-D-glucoside, D-salicin, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, L-rhamnose, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, D-glucose-6-PO_4, D-aspartic acid, D-serine, L-arginine, L-pyroglutamic acid, D-gluconic acid, quinic acid, D-saccharic acid, p-hydroxy phenylacetic acid, D-lactic acid methyl ester, γ-amino-butryric acid, and β-hydroxy-D,L-butyric acid. Major fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$ and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$).

The species includes type strain LMG 31884^T (CCOS 1956^T=GBBC 2123^T) and strains LMG 31887 (CCOS 1955=GBBC 2202), LMG 31885 (CCOS 1954=GBBC 2128) and LMG 31886 (CCOS 1957=GBBC 2199). The strains were isolated from different cultivars of container-grown symptomatic *H. arborescens* plants in the years 2011 and 2012.

Authors and contributors

Conceptualization: NCD, THMS, BC and JFP; data analysis: NCD and JFP; data curation: NCD, JVV, CVM, BC and JFP; funding acquisition: THMS and JFP; investigation: NCD, JVV, CVM, JFP; methodology: NCD, THMS, BC and JFP; resources: JVV, JB, THMS, BC and JFP; software: NCD, JB, and JFP; supervision: JVV, THMS, BC and JFP; validation: NCD, JVV, THMS, BC and JFP; visualization: NCD, THMS, BC and JFP; writing – original draft: NCD, THMS and JFP; writing – reviewing and editing: NCD, JVV, CVM, JB, THMS, BC and JFP.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Funding information

N.C.D. was supported by grant no. IZCOZ0_177064 from the Swiss National Science Foundation (SNSF). This article is based upon work from COST Action CA16107 EuroXanth, supported by COST (European Cooperation in Science and Technology) and upon work from the QBOL Project financed by the 7th Framework Program of the European Union. N.C.D. was granted a Short-Term Scientific Mission by this COST Action to conduct some experiments in Merelbeke (BE).

The EDGAR platform is funded by the BMBF grant FKZ031A533 within the de.NBI network.

T.H.M.S. and J.F.P were supported by the Department of Life Sciences and Facility Management of the Zurich University of Applied Sciences (ZHAW) in Wädenswil, Switzerland. The BCCM/LMG Bacteria Collection is supported by the Federal Public Planning Service – Science Policy, Belgium.

Acknowledgements

The authors would like to thank Marilena Palmisano for valuable laboratory discussions, Nicola Rhyner for his assistance in genomic DNA extraction, Dr Fabio Rezzonico for his assistance with MinION sequencing, Dr Ivana Kroslakova (ZHAW-ICBT) for her support with MALDI-TOF MS, and Christian Kunkel and Dr Gottfried H. Dasen for their assistance and facilitation of strain deposition at CCOS. Special thanks also go to Brigitte De Paepe for her excellent laboratory work in isolating the new *Hydrangea* strains. The authors would also like to thank the HPC team of the School for Life Sciences and Facility Management at ZHAW for their computing resources and support.

References

1. Dowson DW. On the systematic position and generic names of the Gram-negative bacterial plant pathogens. *Zentralblatt Bakteriol Parasitenkd Infekt 2* 1939;100:177-193.

2. Ryan RP, Vorhölter FJ, Potnis N, et al. Pathogenomics of *Xanthomonas*: understanding bacterium–plant interactions *Nat Rev Microbiol* 2011;9:344-355.

3. Vauterin L, Swings J, Kersters K, et al. Towards an improved taxonomy of *Xanthomonas Int J Syst Evol Microbiol* 1990;40:312-316.

4. Lazo GR, Roffey R, Gabriel DW. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism *Int J Syst Evol Microbiol* 1987;37:214-221.

5. Murata N, Starr MP. A concept of the genus *Xanthomonas* and its species in the light of segmental homology of deoxyribonucleic acids *J Phytopathol* 1973;77:285-323.

6. Vauterin L, Hoste B, Kersters K, Swings J. Reclassification of *Xanthomonas Int J Syst Evol Microbiol* 1995;45:472-489.

7. Parkinson N, Aritua V, Heeney J, Cowie C, Bew J, Stead D. Phylogenetic analysis of *Xanthomonas* species by comparison of partial gyrase B gene sequences *Int J Syst Evol Microbiol* 2007;57:2881-2887.

8. Parkinson N, Cowie C, Heeney J, Stead D. Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences *Int J Syst Evol Microbiol* 2009;59:264-274.

9. Young JM, Park DC, Shearman HM, Fargier E. A multilocus sequence analysis of the genus *Xanthomonas Syst Appl Microbiol* 2008;31:366-377.

10. Fischer-Le Saux M, Bonneau S, Essakhi S, Manceau C, Jacques M-A. Aggressive emerging pathovars of *Xanthomonas arboricola* represent widespread epidemic clones distinct from poorly pathogenic strains, as revealed by multilocus sequence typing *Appl Environ Microbiol* 2015;81:4651-4668.

11. Timilsina S, Kara S, Jacques MA, et al. Reclassification of *Xanthomonas gardneri* (ex Šutič 1957) Jones et al. 2006 as a later heterotypic synonym of *Xanthomonas cynarae* Trébaol et al. 2000 and description of *X. cynarae* pv. cynarae and *X. cynarae* pv. gardneri based on whole genome analyses *Int J Syst Evol Microbiol* 2019;69:343-349.

12. Morinière L, Burlet A, Rosenthal ER, et al. Clarifying the taxonomy of the causal agent of bacterial leaf spot of lettuce through a polyphasic approach leads to combine *Xanthomonas hortorum* Vauterin et al. 1995 and *Xanthomonas cynarae* Trébaol 2000 emend. Timilsina et al. 2019 *Syst Appl Microbiol* Published online 2020:126087.

13. Trébaol G, Gardan L, Manceau C, Tanguy JL, Tirilly Y, Boury S. Genomic and phenotypic characterization of *Xanthomonas cynarae* sp. nov., a new species that causes bacterial bract spot of artichoke (*Cynara scolymus* L.) *Int J Syst Evol Microbiol* 2000;50:1471-1478.

14. Jones JB, Lacy GH, Bouzar H, Stall RE, Schaad NW. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper *Syst Appl Microbiol* 2004;27:755-762.

15. EU Commission. *Commission Implementing Directive (EU), 2020. Amending Council Directives 66/401/EEC, 66/402/EEC, 68/193/EEC, 2002/55/EC, 2002/56/EC and 2002/57/EC, Commission Directives 93/49/EEC and 93/61/EEC and Implementing Directives 2014/21/EU and 2014/98/EU as Regards Pests of Plants on Seeds and Other Plant Reproductive Material. Official Journal L41, 1-77.*; 2020.

16. Kendrick JB. Bacterial blight of carrot *J Agric Res* 1934;49:493-510.

17. Brown NA. Bacterial leaf spot of geranium in the eastern United States. *J Agric Res* 1923;23:361-372.

18. Niederhauser JS. A bacterial leaf spot and blight of the Russian dandelion *Phytopathology* 1943;33:959-961.

19. Arnaud G. Une maladie bactérienne du lierre (*Hedera helix* L.) *Comptes Rendus Hebd Séances Académie Sci* 1920;171:121-122.

20. Brown NA. Some bacterial diseases of lettuce *J Agric Res* 1918;13:367-388.

21. Cottyn B, De Paepe B, Dia NC, Van Malderghem C, Pothier JF, Van Vaerenbergh J. First report of bacterial leaf spot of *Hydrangea* in retail nurseries in Flanders (Belgium) caused by strains forming a new *Xanthomonas hortorum* clade *New Dis Reps* 2021;43:e120008.

22. Garita-Cambronero J, Palacio-Bielsa A, López MM, Cubero J. Comparative genomic and phenotypic characterization of pathogenic and non-pathogenic strains of *Xanthomonas arboricola* reveals insights into the infection process of bacterial spot disease of stone fruits *PLoS ONE* 2016;11.

23. Dia NC, Rezzonico F, Smits THM, Pothier JF. Complete or high-quality draft genome sequences of six *Xanthomonas hortorum* strains sequenced with short- and long-read technologies *Microbiol Resour Announc* 2020;9.

24. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms *Mol Biol Evol* 2018;35:1547-1549.

25. Hauben L, Vauterin L, Swings J, Moore ER. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species *Int J Syst Bacteriol* 1997;47:328-335.

26. Glaeser SP, Kämpfer P. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy *Syst Appl Microbiol* 2015;38:237-245.

27. Nei M, Kumar S. *Molecular Evolution and Phylogenetics* Oxford University Press; 2000.

28. Meier-Kolthoff JP, Göker M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy *Nat Commun* 2019;10:1-10.

29. Lefort V, Desper R, Gascuel O. FastME 2.0: a comprehensive, accurate, and fast distance-based phylogeny inference program *Mol Biol Evol* 2015;32:2798-2800.

30. Farris JS. Estimating phylogenetic trees from distance matrices *Am Nat* 1972;106:645-668.

31. Kreft Ł, Botzki A, Coppens F, Vandepoele K, Van Bel M. PhyD3: a phylogenetic tree viewer with extended phyloXML support for functional genomics data visualization *Bioinformatics* 2017;33:2946-2947.

32. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries *Nat Commun* 2018;9:1-8.

33. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions *BMC Bioinform* 2013;14:60.

34. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition *Proc Natl Acad Sci* 2009;106:19126-19131.

35. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison *Bioinformatics* 2016;32:929-931.

36. Blom J, Kreis J, Spänig S, et al. EDGAR 2.0: an enhanced software platform for comparative gene content analyses *Nucleic Acids Res* 2016;44:W22-W28.

37. LAMP: Design primers for Loop-Mediated Isothermal Amplification. Accessed August 16, 2020. http://www.premierbiosoft.com/isothermal/lamp.html

38. Blaser S, Diem H, Felten A von, et al. From laboratory to point of entry: development and implementation of a loop-mediated isothermal amplification (LAMP)-based genetic identification system to prevent introduction of quarantine insect species *Pest Manag Sci* 2018;74:1504-1512.

Table 1. Isolation information of *Xanthomonas hydrangea* strains used in this study.

Table 2. Selected differential phenotypic characteristics of *Hydrangea* strains and its closest phylogenetic relative *Xanthomonas hortorum* characterized in triplicates using Biolog GEN III MicroPlates.

Table 3. Genome metrics of *Xanthomonas hydrangea* strains.

Table 4. LAMP primer set *Xhyd*-B used for the identification and detection of *Xanthomonas hydrangea*.

Fig. 1. Symptoms at 28 days post inoculation (a) on *Hydrangea arborescens* cv. Annabelle leaves inoculated by pressure-spraying with *Xanthomonas hydrangeae* strain LMG 31884^T (= CCOS 1956^T, GBBC 2123^T) and (b) on *Hydrangea quercifolia* leaves inoculated with *Xanthomonas hydrangeae* strains LMG 31884^T (= CCOS 1956^T, GBBC 2123^T) (left) and LMG 31885 (= CCOS 1954, GBBC 2128) (right).

Fig. 2. Maximum-likelihood tree based on partial 16S rRNA gene sequences, constructed using the General Time Reversible model. There were a total of 1,227 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. *Xanthomonas hydrangeae* strains are in bold font and strains of the *Xanthomonas hortorum* species level clade are marked with rectangle boxes. The 16S rRNA gene sequence and contig accessions are noted in grey. When no 16S rRNA gene accession is available, contig accession in addition to the gene's position on the contig is reported between brackets. Percent bootstrap support values calculated for 1,000 iterations are indicated near nodes only when over 51.

Fig. 3. Maximum-likelihood tree based on MLSA using concatenated sequences of *atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, *lepA* and *rpoD* (total 11,415 bp). The tree highlights the phylogenetic relationship of *Xanthomonas hydrangeae* LMG 31884^T, LMG 31887, LMG 31885 and LMG 31886 (bold, circle), in relation to *Xanthomonas hortorum* subclusters A and B, marked with a diamond and a square, respectively. The *X. hortorum* species level clade is marked with an arrow. Percent bootstrap support values calculated for 1,000 iterations are indicated near nodes only when over 51.

Fig. 4. Whole-genome phylogeny of *Xanthomonas hydrangeae* LMG 31884^T, LMG 31887, LMG 31885 and LMG 31886 (bold, circle). The *Xanthomonas hortorum* species level clade is marked with a triangle, and *X. hortorum* subclusters A and B are marked with a diamond and a square, respectively. The phylogeny was inferred from the genome BLAST distance

588 phylogeny approach (GBDP). The approach consists of two steps: a BLAST genome
589 comparison followed by distance matrix computation to construct a phylogenetic tree.

590 **Fig. 5.** Bi-directional table showing average nucleotide identity (fastANI, upper half of table)
591 and *in silico* DNA-DNA hybridization (*is*DDH, lower half of table) values between
592 *Xanthomonas hydrangeae*, its closest phylogenetic relative *Xanthomonas hortorum* and
593 three outgroup strains, *Xanthomonas populi*, *Xanthomonas arboricola* and *Xanthomonas*
594 *campestris*.

595 **Table 1.** List of *Xanthomonas hydrangeae* strains used in this study.

Strain*	Isolation country	Isolation host	Isolation year
LMG 31884^T (CCOS 1956^T, GBBC 2123^T)	Belgium	*Hydrangea arborescens* cv. Bella Anna	2011
LMG 31887 (CCOS 1955, GBBC 2202)	Belgium	*Hydrangea arborescens* cv. Invincibelle	2012
LMG 31885 (CCOS 1954, GBBC 2128)	Belgium	*Hydrangea arborescens* cv. Bella Anna	2011
LMG 31886 (CCOS 1957, GBBC 2199)	Belgium	*Hydrangea arborescens* cv. Invincibelle	2012

596

597 * Abbreviations: BCCM/LMG: the Belgian Coordinated Collections of Microorganisms/
598 bacteria collection Laboratory of Microbiology at Ghent University, Belgium; CCOS: Culture
599 Collection of Switzerland in Wädenswil, Switzerland; GBBC: Gewasbescherming Bacteriële
600 Collectie (English: Plant Health Unit Bacterial Collection) at ILVO, Merelbeke, Belgium.

Table 2. Selected differential phenotypic characteristics of *Hydrangea* strains and its closest phylogenetic relative *Xanthomonas hortorum* characterized in triplicates using Biolog GEN III MicroPlates.

The substrates that can distinguish the *Hydrangea* strains from *X. hortorum* are indicated in bold. The complete results for the biochemical panels tested below are listed in Table S3. Strains: 1: LMG 31884^{T}; 2: LMG 31885; 3: *X. hortorum* pv. pelargonii CFBP 2533^{PT}; 4: *X. hortorum* pv. carotae CFBP 7900; 5: *X. hortorum* pv. hederae CFBP 4925^{T}; 6: *X. hortorum* pv. taraxaci NCPPB 940^{PT}; 7: *X. hortorum* pv. vitians LMG 938^{neoPT}; 8: *X. hortorum* pv. cynarae CFBP 4188^{PT}; 9: *X. hortorum* pv. gardneri ATCC 19865^{PT}; 10: *X. campestris* pv. campestris ATCC 33913^{T}.

Characteristic	**1**	**2**	**3***	**4***	**5***	**6***	**7***	**8***	**9***	**10**†
Utilization of:										
Dextrin	w‡	w	-	-	w(-)	-	-	-	-	+
β-Gentiobiose	v	+	+	+	-(+)	+	+	-	-	+
D-Raffinose	-	-	-	-	+(-)	-	-	-	-	V+
α-D-Lactose	-(+)	+	-	-	+(-)	-	+(-)	-	+	-
3-Methyl Glucose	w(-)	-	-	-	-	-	-	-	-	NA
D-Fucose	w	w(-)	-	-	w(-)	-	-	-	-	NA
Inosine	w	w(-)	-	-	-	-	-	-	-	V-
Glycerol	+	+	+	+	+	+	+	+	-	V
D-Fructose-6-PO$_4$	**w**	**w**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**NA**
Glycyl-L-Proline	+	+	+	+	+	+	+	+	v	V-
L-Alanine	+	+	+	+	+	+	+	w	+	V+
L-Aspartic Acid	+	w(+)	-	+	w(-)	w(-)	v	w(-)	+	V-
L-Histidine	v	+	+	-	+	+	-(+)	w(+)	+	-
Pectin	+	+	+	+	+	+	+	-	+	NA
D-Galacturonic Acid	w	w(-)	-	-	-	-	-	-	-	-
L-Galactonic Acid Lactone	w	-	-	-	-	-	-	-	-	-
D-Glucuronic Acid	w	w	-	-	w(-)	-	-	-	-	-
Glucuronamide	**w**	**w**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Mucic Acid	w(-)	-	-	-	-	-	-	-	-	NA

L-Lactic Acid	+	+	+	w(-)	+	+(-)	+	w	+	V+
D-Malic Acid	w(-)	w	-	-	-	-	-	-	-	NA
Bromo-Succinic Acid	+	+	w(+)	+	+	+	+	+	+	+
Tween 40	+	+	w(+)	+	w	w(+)	+	w(-)	+	V+
α-Hydroxy-Butyric Acid	+	w(+)	-	w(-)	w(-)	v	-	-	w(-)	-
α-Keto-Butyric Acid	+	w	-	+(-)	+	-(+)	+	v	+	V+
Acetoacetic Acid	+	w(+)	w(+)	-	+	+	w(+)	w	+	NA
Formic Acid	+	w	+	+(-)	-(+)	+(-)	w(+)	+(-)	+	-
Growth in presence of:										
pH 5	w(-)	-	-	-(+)	-	-	-	-(+)	-	NA
4% NaCl	w	w	+	+	w(-)	-	+	v	+	NA
8% NaCl	-	-	-	-	-	-	-(+)	-	-	NA
Fusidic Acid	w(-)	-	-	-	-	-	-	-	-	NA
D-Serine	w(-)	-	-	-	-	-	+(-)	-	-	-
Troleandomycin	w(-)	-	-(+)	v	-	-	-	-	-	NA
Rifamycin SV	+	+	-	+	-(+)	-(+)	-(+)	+(-)	-	NA
Minocycline	w(-)	-	-	-	-	-	-	-	-	NA
Guanidine hydrochloride	w	w(-)	+(-)	+	+	+(-)	+(-)	+	+(-)	NA
Niaproof 4	w	w	+(-)	+	w(-)	v	w(-)	v	-	NA
Vancomycin	w(+)	w	v	w(-)	-	-	-	-	-	NA
Tetrazolium Violet	+	+	+	+	-(+)	+(-)	+	+(-)	+	NA
Tetrazolium Blue	+	+	+	+	+	+(-)	+	+	+	NA
Nalidixic Acid	w(-)	-	-	-	-	-	-	-	-	NA
Lithium Chloride	**-**	**-**	**+(-)**	**+**	**+(-)**	**-(+)**	**+(-)**	**+(-)**	**w(-)**	**NA**
Potassium Tellurite	-	-	+	+	w(+)	-	+	-	-	NA
Aztreonam	+	w(-)	+	+	+	+	+	+	+(-)	NA
Sodium Butyrate	w(-)	-	-	-	-	-	-	-	-	NA
Sodium Bromate	-	-	+	v	-	-	w(+)	v	w(-)	NA

607

608 * Phenotypic profiles of subclusters A and B of *X. hortorum* were obtained from Morinière *et al.* [12].

609 † Phenotypic profiles of *X. campestris* pv. campestris ATCC 33913^T were adapted from Vauterin *et al.* [6] based on the profiles of homology
610 group 15: + or – indicate unequivocally positive or negative results, respectively, while V+ or V- indicate activities in more or less than 50% of
611 tested strains within homology group 15. Non-tested strains are noted with “NA”.

612 ‡ Phenotypes were categorized as positive (+), weak (w) or negative (-). Triplicates were managed in the same way as described by Morinière
613 *et al.* [12] to ensure comparability of results: + = (+/+/+) or (+/+/w), w = (w/w/w), − = (−/−/−) or (−/−/w), +(−) = (+/+/−), −(+) = (−/−/+), w(+) =
614 (w/w/+), w(−) = (w/w/−), v = (−/w/+).

615

616

Table 3. Genome metrics of *Xanthomonas hydrangeae* strains.

Strain	Genome length in bp (status)	G+C content (mol%)	CDS no.	No. of contigs	Genome composition	EMBL accession no.	EMBL assembly accession no.
LMG 31884^T (= CCOS 1956^T, GBBC 2123^T)	5,578,501 (complete)	63.6	4,703	5	Chromosome: 5,359,476 bp pLMG31884_p76: 76,708 bp pLMG31884_p73: 73,155 bp pLMG31884_p46: 46,175 bp pLMG31884_p23: 22,987 bp	LR990730 LR990731 LR990732 LR990733 LR990734	GCA_905142475
LMG 31887 (= CCOS 1955, GBBC 2202)	5,466,350 (complete)	63.7	4,580	3	Chromosome: 5,352,937bp pLMG31887_p67: 67,235 bp pLMG31887_p46: 46,178 bp	LR990736 LR990737 LR990738	GCA_905142485
LMG 31885 (= CCOS 1954, GBBC 2128)	5,357,374 (high-quality draft)	63.9	4,452	3	Chromosome: 5,243,961 bp* pLMG31885_p67: 67,237 bp pLMG31885_p46: 46,176 bp	LR990741 LR990742 LR990743	GCA_905142495
LMG 31886 (= CCOS 1957, GBBC 2199)	5,297,786 (complete)	63.9	4,388	2	Chromosome: 5,251,608 bp pLMG31886_p46: 46,179bp	LR990739 LR990740	GCA_905142465

* Non-circularized chromosome.

619 **Table 4.** LAMP primer set *Xhyd*-B used for the detection of *Xanthomonas hydrangea.*

Primer name	Sequence (5'-3')
Xhyd-B-F3	GCGAAGTTATTGCTAACACG
Xhyd-B-B3	CGTTCGGCTGTAACTTGT
Xhyd-B-FIP	TTGGAGCGAATCAGCCGACTTTTTGATCGGTGTGAGTAGCA
Xhyd-B-BIP	AAGCTCTGCTGACGCCACTTTTCCAAATCATCTGGCGTAAATG
Xhyd-B-LoopF	ATGTAGGCATACCACGATTCAA
Xhyd-B-LoopB	CCTGCCGATTGGGTTAAGT

620