INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

López *et al.*, *Int J Syst Evol Microbiol* 2018;68:1857–1866

DOI 10.1099/ijsem.0.002743

MICROBIOLOGY SOCIETY

Xanthomonas prunicola sp. nov., a novel pathogen that affects nectarine (*Prunus persica* var. *nectarina*) trees

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Abstract

Three isolates obtained from symptomatic nectarine trees (*Prunus persica* var. *nectarina*) cultivated in Murcia, Spain, which showed yellow and mucoid colonies similar to *Xanthomonas arboricola* pv. *pruni*, were negative after serological and real-time PCR analyses for this pathogen. For that reason, these isolates were characterized following a polyphasic approach that included both phenotypic and genomic methods. By sequence analysis of the 16S rRNA gene, these novel strains were identified as members of the genus *Xanthomonas*, and by multilocus sequence analysis (MLSA) they were clustered together in a distinct group that showed similarity values below 95 % with the rest of the species of this genus. Whole-genome comparisons of the average nucleotide identity (ANI) of genomes of the strains showed less than 91 % average nucleotide identity with all other species of the genus *Xanthomonas*. Additionally, phenotypic characterization based on API 20 NE, API 50 CH and BIOLOG tests differentiated the strains from the species of the genus *Xanthomonas* described previously. Moreover, the three strains were confirmed to be pathogenic on peach (*Prunus persica*), causing necrotic lesions on leaves. On the basis of these results, the novel strains represent a novel species of the genus *Xanthomonas*, for which the name *Xanthomonas prunicola* is proposed. The type strain is CFBP 8353 (=CECT 9404=IVIA 3287.1).

The genus *Xanthomonas* currently contains 31 bacterial species which are responsible for diseases in a broad range of economically important crops, such as stone-fruit trees, citrus and rice, and also in wild plants [1]. The taxonomy of this genus is always under revision. The first classification of members of the genus *Xanthomonas* was based on host specificity, according to the 'new host–new species' concept, leading to a complex genus with more than 100 species [2, 3]. Dye and Lelliott [4] reduced the number of species of the genus *Xanthomonas* to only five (*Xanthomonas albilineans*, *Xanthomonas ampelina*, *Xanthomonas axonopodis*, *Xanthomonas campestris* and *Xanthomonas fragariae*) and included the remaining nomenspecies in the single species *X. campestris*. Later, Young *et al.* [5] proposed renaming the former nomenspecies as pathovars of *X. campestris*. A pathovar is an infrasubspecific taxonomic range, grouping strains with the same or similar characteristics, responsible for the same symptoms in the same host range [6], and since then pathovar classification has been commonly used by all phytopathologists.

Based on species definition [7, 8], it is mandatory to delineate novel species using a polyphasic approach including both phenotypic and genotypic characters. Phenotypic methods such as conventional biochemical [9] or

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Keywords: ANI; phenotypic characterization; polyphasic approach; MLSA; new species; *Prunus*.
Abbreviations: AIC, Akaike Information Criterion; ANI, Average Nucleotide Identity; ANIb, Average Nucleotide Identity based on the blast algorithm; BLAST, Basic Local Alignment Search Tool; FAME, Fatty Acid Methyl Esters; HR, Hypersensitivity Reaction; ML, Maximum Likelihood; MLSA, Multilocus Sequence Analysis; NTSYS, Numerical Taxonomy System; RDP, Ribosomal Database Project; Rep-PCR, Repetitive Element Palindromic PCR.
†These authors contributed equally to this work.
The GenBank accession numbers for the sequences of three strains (CFBP 8353, CFBP 8354 and CFBP 8355) are MG523312, MG523313 and MG523314 for the 16S rRNA gene, MG570215, MG570214 and MG570213 for the *dnaK* gene, MG570218, MG570217 and MG570216 for the *fyuA* gene, MG601514, MG601513 and MG601512 for the *gyrB* gene and MG570221, MG570220 and MG570219 for the *rpoD* gene, respectively. The GenBank accession numbers for the genome sequences are PHKV00000000, PHKW00000000 and PHKX00000000 for CFBP 8553, CFBP 8554 and CFBP 8555, respectively.
Strains can be retrieved from CIRM-CFBP (https://www6.inra.fr/cirm_eng/CFBP-Plant-Associated-Bacteria; DOI 10.15454/1.5103266699001077E12) and CECT (www.cect.org).
One supplementary table is available with the online version of this article.

pathogenicity tests [10], fatty acid analysis [11] and protein profiling [12], among others, have been widely applied in taxonomic studies of the *Xanthomonas* group. In addition, molecular methods have also been used, including plasmid profiling [13], repetitive-sequence-based PCR (rep-PCR) [14] and especially DNA–DNA hybridization [15], which was considered the 'gold standard' to delimit bacterial species [16]. However, this technique has several drawbacks that limit its use, since it is labour-intensive and time-consuming and, above all, because of the impossibility of building incremental databases, in contrast to sequence information [17]. Therefore, alternative methods like multilocus sequence analysis (MLSA) [18] and especially the average nucleotide identity (ANI) between a given pair of genomes [19] are currently the most widely utilized techniques to delineate novel bacterial species.

The major *Xanthomonas* reclassification was accomplished by Vauterin *et al.* [15], who described 20 species of the genus *Xanthomonas* based on DNA–DNA hybridization and physiological tests. Among them, *Xanthomonas arboricola* was defined as a novel species that comprised six pathovars (*celebensis*, *corylina*, *fragariae*, *juglandis*, *populi* and *pruni*). The pathovar *pruni* regrouped the strains affecting species of the genus *Prunus*, mainly stone fruits and almond, but also some ornamentals. In subsequent years, further species of the genus have been identified, such as *Xanthomonas cynarae* [20], *Xanthomonas euvesicatoria*, *Xanthomonas gardneri*, *Xanthomonas perforans* [21], *Xanthomonas alfalfae*, *Xanthomonas citri, Xanthomonas fuscans*, [22], *Xanthomonas dyei* [23], *Xanthomonas maliensis* [24] and the recently described *Xanthomonas floridensis* and *Xanthomonas nasturtii* [25]. None of these species have been reported as affecting species of the genus *Prunus*.

In our study, bacterial isolates were obtained from canker exudates of symptomatic nectarine trees. The symptoms observed were quite similar to those of the bacterial spot disease of stone fruits produced by *X. arboricola* pv. *pruni*. Bacterial isolates from the affected plants showed morphological features similar to those of *X. arboricola* pv. *pruni*. After phenotypic and molecular characterization, the novel strains were confirmed as representing members of the genus *Xanthomonas*, but many of their characteristics differed from those of any of the other species of this genus described previously. The objective of this work was to characterize such atypical strains through a polyphasic approach including pathogenicity and phenotypic tests, sequence analysis of the 16S rRNA gene, MLSA targeting the housekeeping genes *dnaK*, *fyuA*, *gyrB* and *rpoD*, and comparative whole-genome analysis based on ANI.

BACTERIAL STRAINS AND GROWTH CONDITIONS

Three novel isolates of members of the genus *Xanthomonas* (IVIA 3287.1, IVIA 3287.2 and IVIA 3287.3) were obtained in 2007 from symptomatic nectarines (*Prunus persica* var. *nectarina*) of the cultivar Lourdes in Murcia (Spain). They were recovered from trunk canker exudates of the trees after three days incubation at 25 °C on yeast peptone glucose agar (YPGA) medium. The colonies were purified three times in YPGA and deposited in bacterial collections with accession numbers IVIA 3287.1 (=CFBP 8353=CECT 9404), IVIA 3287.2 (=CFBP 8354=CECT 9405) and IVIA 3287.3 (=CFBP 8355=CECT 9406).

These bacterial isolates were characterized together with 20 strains of *X. arboricola* pv. *pruni* and 10 strains of other species of the genus *Xanthomonas* (Table S1, available in the online version of this article). Prior to performing the analyses, the bacteria were grown alternatively on YPGA medium or Luria–Bertani (LB) agar or trypticase soy broth agar (TSBA), under different conditions depending on the test.

PATHOGENICITY TESTS

Pathogenicity of the three strains was tested following two different methodologies. First, inoculation on detached leaves of seedlings of peach GF 305 (*Prunus persica*) was performed in accordance with the protocol of Randhawa and Civerolo [26]. Briefly, bacterial suspensions adjusted to 10^6 c.f.u. ml^{-1} were prepared in 10 mM phosphate buffered saline (PBS) from 48 h cultures grown on YPGA medium. The leaves were briefly washed under running tap water and disinfected for 40–60 s with 70 % ethanol. They were rinsed repeatedly in sterile water. Suspensions were then used to inoculate the leaves on the abaxial side. Inoculations were performed by infiltration using a syringe without a needle and applying gentle and steady pressure until the mesophyll tissue was water-soaked. Following this, the leaves were placed on 0.5 % water agar plates and incubated at 28 °C. Three leaves per strain were inoculated in two independent experiments. The presence or absence of symptoms was recorded from 5 to 10 days post inoculation (p.i.). Second, novel strains were also inoculated in one-year-old GF-305 plants cultivated in a greenhouse. Six leaves were inoculated per plant, three on the abaxial side by infiltration, as described for the detached leaves, and three on the adaxial side, by infiltration of bacterial suspensions at 10^8 c.f.u. ml^{-1} into the mesophyll using a syringe fitted with a needle. Two plants were inoculated with each strain. Plants were incubated in a growth chamber at 30 °C and a photoperiod of 16 h, and the presence or absence of symptoms was evaluated through 28 days p.i. In both assays, negative and positive controls were included using sterile distilled water and a suspension of *X. arboricola* pv. *pruni* strain ISPAVE B4, respectively. Additionally, leaves of tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*) plants were inoculated with bacterial suspensions of the novel strains at 10^8 c.f.u. ml^{-1} to test the hypersensitivity reaction (HR), in accordance with the methods of Schaad and Stall [27].

On detached leaves of GF 305 peach plants, the novel strains produced similar symptoms to those caused by *X. arboricola* pv. *pruni*. On the adaxial side of the leaves, brown spots were observed at 7 days p.i., which evolved to bigger dark

lesions after 10 days p.i. Some exudates were also observed on the abaxial surface. In whole plants of GF-305 inoculated with novel strains, small necrotic spots were observed at 5 days p.i. with both inoculation procedures. These lesions expanded on the following days. Some leaves showed severe necrotic lesions at 12 days p.i. causing leaf dieback in some cases, thereby confirming their pathogenicity on species of the genus *Prunus*. Quite similar symptoms were observed on detached leaves and whole plants inoculated with the *X. arboricola* pv. *pruni* strain ISPAVE B4, but not in the negative controls. Colonies showing the same morphological type as the inoculated strains were recovered from the diseased tissues. Finally, the three novel strains obtained from nectarine were able to elicit an HR on tomato and tobacco leaves after 24 and 72 h, respectively.

SEROLOGICAL TESTS AND PCR ANALYSIS

Since novel bacterial strains were obtained from plants of the genus *Prunus*, standard serological tests and PCR protocols used for *X. arboricola* pv. *pruni* identification were initially performed in order to identify the novel strains. Two different serological tests were utilised, first, indirect immunofluorescence based on a polyclonal antibody obtained from *X. arboricola* pv. *pruni* (As69V2) used at different concentrations (1 : 1000, 1 : 2000, 1 : 4000 and 1 : 5000), including *X. arboricola* pv. *pruni* strain ISPAVE B4 as a positive control [28]. Second, analysis with a prototype of lateral flow immunoassay recently developed for the detection of *X. arboricola* pv. *pruni* using two other polyclonal antibodies generated from this pathogen (2626.1-WC and 2626.1-HT) [29]. Negative results were obtained by both methodologies for the three strains.

PCR analyses were performed following two protocols used for *X. arboricola* pv. *pruni* detection and identification. Bacterial DNA was extracted from pure cultures obtained after 48 h incubation in YPGA medium at 25 °C, using a simple DNA extraction method [30]. Real-time PCR protocols using primers targeting an ABC transporter protein coding gene [31] and the virulence effector XopE3 [32] were performed. In all the reactions, *X. arboricola* pv. *pruni* strain ISPAVE B4 was used as a positive control. No amplification was observed with either of the two protocols for the novel strains.

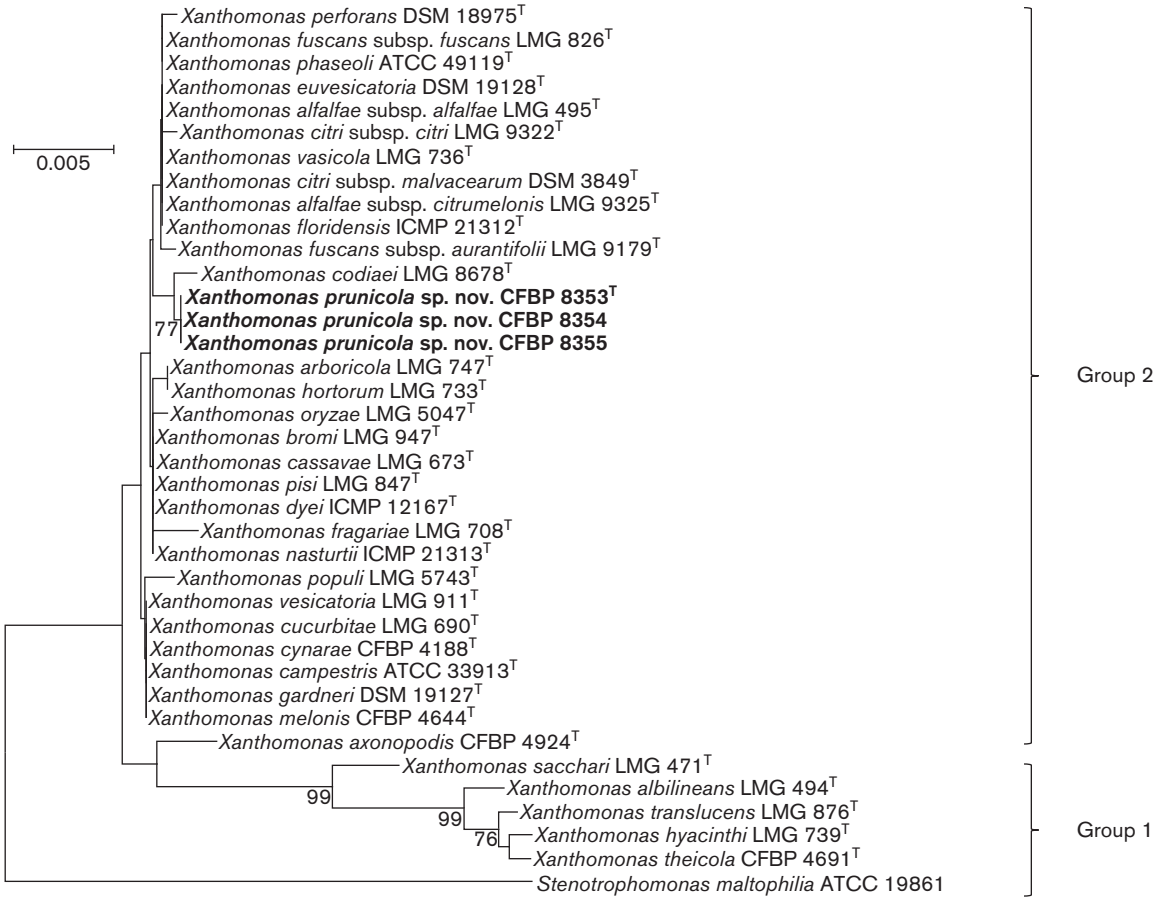

Fig. 1. Neighbour-joining phylogenetic tree reconstructed from 16S rDNA sequences of type strains of species of the genus *Xanthomonas*. Bootstrap values (100 replicates) higher than 50 % are displayed. The newly isolated strains display similar sequences, belonging to group 2 of the genus *Xanthomonas* [18].

Results obtained from serological tests and real-time PCR analysis confirmed that the strains that were pathogenic to nectarine could not be considered as typical strains of *X. arboricola* pv. *pruni*.

SEQUENCE ANALYSIS OF 16S rRNA GENE

Complete 16S rRNA gene sequences of the three novel strains were retrieved from the draft genomic sequences, as mentioned later, by BLASTn searches [33]. 16S rRNA genes of the novel strains were aligned with those of the type strains of the species retrieved from the RDP database (http://rdp.cme.msu.edu/) or from the draft genomic sequences of CFBP 4644^T (*Xanthomonas melonis*), CFBP 4691^T (*Xanthomonas theicola*) and CFBP 4924^T (*X. axonopodis*) [34]. Alignment (using ClustalW) and phylogenetic tree reconstruction (using the neighbour-joining method; 100 bootstrap re-samplings) were conducted using MEGA 6.0 [35].

The 16S rRNA sequences from the three novel strains were 100 % identical. BLASTn analysis on NCBI showed that these three strains clearly represent members of the genus *Xanthomonas* and share 99 % similarities with other species of the genus *Xanthomonas* (*Xanthomonas vesicatoria*, *Xanthomonas oryzae*, *Xanthomonas pisi*, *X. citri*, *X. campestris*, *X. arboricola*) (results not shown). Phylogenetic analysis of the 16S sequences of type strains of members of the genus *Xanthomonas* revealed that these strains belonged to group 2 of the genus (Fig. 1). This group was defined by Young *et al.* [18].

MLSA

A multilocus sequence analysis based on the partial sequences of the *dnaK*, *fyuA*, *gyrB* and *rpoD* genes, which have been previously determined as useful for characterizing novel species of the genus *Xanthomonas* [18, 23], was performed. PCR amplifications were carried out in a 50 µl volume containing 1× PCR buffer [10 mM Tris-HCl, 50 mM KCl, 0.1 % Triton X-100 (pH 9.0)]; 0.2 µM of each primer; 1.25 U Taq DNA polymerase (Biotools); 0.2 mM each dNTP (Biotools); 1.5 mM $MgCl_2$ and 1.0 µg µl^{-1} of DNA template. All PCR reactions were performed in an ABI 2720 thermal cycler (Applied Biosystems) with an initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCR products were sequenced at STAB VIDA (Lisbon, Portugal), and edited using BioEdit Sequence Alignment Editor [36]. Sequences of the four housekeeping genes used for MLSA analysis of the species of the genus *Xanthomonas* that belong to phylogenetic group 2 [18] were obtained from the National Centre for Biotechnology Information database (NCBI) (http://www.ncbi.nlm.nih.gov) and included in the analysis. Sequences obtained were trimmed to the following sizes: *dnaK*, 890 positions; *fyuA*, 653 positions; *gyrB*, 848 positions and *rpoD*, 843 positions. Sequences were then concatenated to give a total length of 3234 nucleotide positions. ClustalW version 1.83 [37] was used for all alignments and the program MEGA 6.0 [35] was used to determine the best model of evolution for maximum-likelihood (ML) analysis based on the Akaike information criterion (AIC) [38]. For the concatenated gene dataset, the TN93+G model was selected and maximum-likelihood trees, using 1000 bootstrap re-samplings, were generated. A similarity matrix of the concatenated sequences of the novel strains and all the other species of the genus *Xanthomonas* was calculated with MEGA 6.0.

The results of ML analysis of the concatenated nucleotide sequences (3234 nucleotide positions) indicated that all the strains representing the species of the genus *Xanthomonas* according to Young *et al.* [18] were grouped in agreement with the taxonomic classification of the species of the genus. Bootstrap values for most species branch nodes exceeded 90 %. The three novel strains showed identical concatenated sequences and were clustered together into a distinct group separated from the other species described in the genus *Xanthomonas* (Fig. 2) (Table 1). The mean nucleotide similarity among the group of the novel strains and the known species of the genus *Xanthomonas* was 93.4±0.3 % and the most closely related species were *X. vasicola* pv. *holcicola* and *X. oryzae* pv. *oryzicola*, which had similarity values of 94.98±0.34 and 94.64±0.35 %, respectively (Table 1). All the similarity values obtained between the group of the novel strains and the other species of the genus *Xanthomonas* were lower than 96 %, which is the cut-off point proposed by Young *et al.* [18] for species differentiation in this genus, thus confirming this group of strains as representing a novel species of the genus *Xanthomonas*.

COMPARATIVE WHOLE-GENOME ANALYSIS BASED ON AVERAGE NUCLEOTIDE IDENTITY (ANI)

Genomic DNA of strains CFBP 8353, CFBP 8354 and CFBP 8355 was extracted using the Wizard Genomic DNA purification kit (Promega). Whole genomes were sequenced using Illumina technology on a MiSeq instrument. Libraries of genomic DNA were performed using the Nextera XT kit (Illumina). Paired-end reads of 2×100 bp were assembled in contigs using SOAPdenovo 1.05 [39] and Velvet 1.2.02 [40]. Annotation was performed using EuGene-PP [41] with the genome of strain Xoc BLS256 from *X. oryzae* pv. *oryzicola* as a reference.

Genomic DNA of strains CFBP 8353, CFBP 8354 and CFBP 8355 has a total length of 5.32, 5.31 and 5.34 Mb, respectively, and contains 95, 77 and 32 contigs, respectively.

Draft genomes of the novel strains were compared with each other and with those of the most closely phylogenetically related type strains identified by MLSA. The average nucleotide identity based on the blast algorithm (ANIb) was calculated for each pairwise comparison using Jspecies software [19]. By definition, two strains having an ANIb value above the 95 % threshold represent the same species [19]. Draft genomes were deposited in GenBank with the following accession numbers PHKV00000000, PHKW00000000

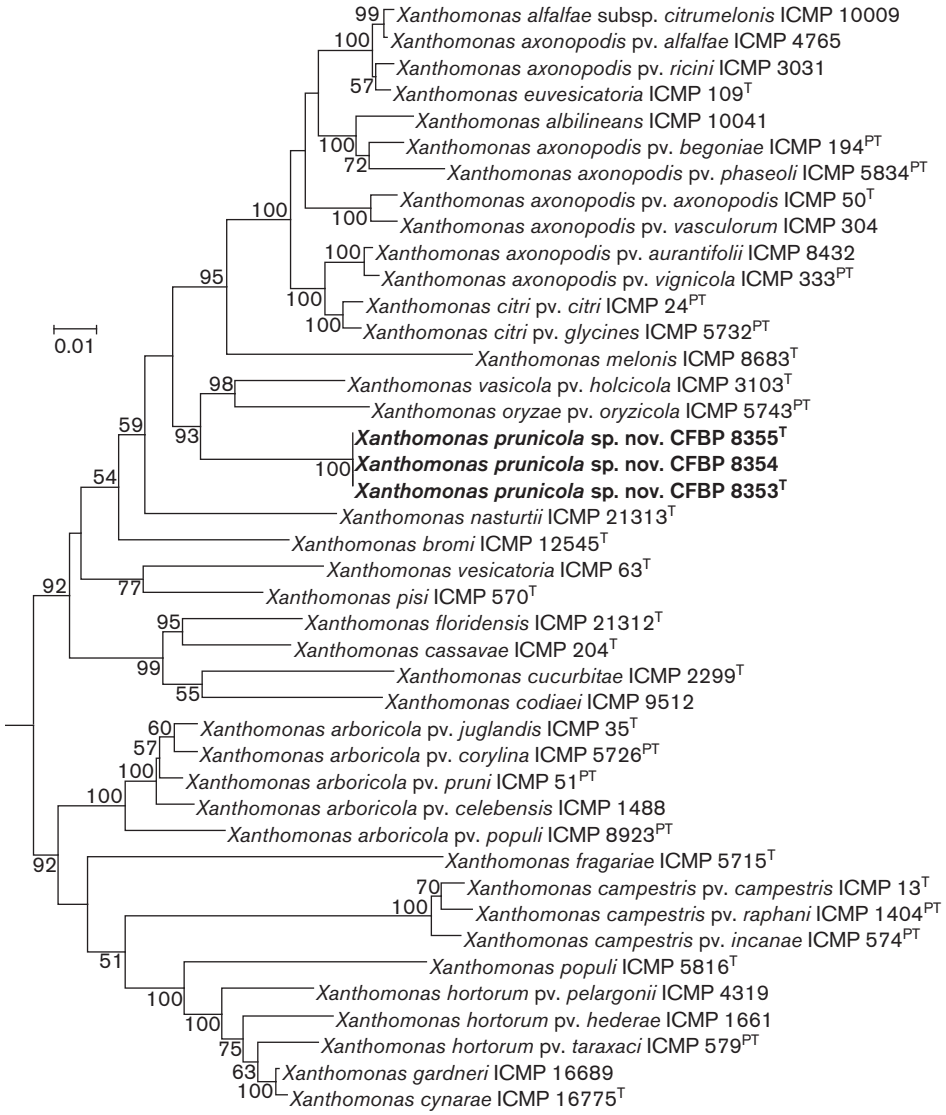

Fig. 2. Maximum likelihood analysis of the concatenated partial nucleotide sequences of the *dnaK*, *fyuA*, *gyrB* and *rpoD* genes representing the phylogenetic position of the novel isolates within the genus *Xanthomonas*. Novel isolates appear in bold type and the remaining strains are a selection of reference strains of members of the genus *Xanthomonas* reported in Young *et al*. [18] and Vicente *et al*. [25]. Bootstrap values (1000 replicates) are shown over or below the branches.

and PHKX00000000 for CFBP 8553, CFBP 8554 and CFBP 8555, respectively.

ANIb values among novel strains were higher than 99.95 %, thus confirming that the three strains should be classified as members of the same species. All ANIb values in pairwise comparisons with type strains of already described species were far below the 95 % delineation cut-off. In accordance with the MLSA results, the highest ANIb values were obtained with *X. oryzae* (91.13–91.14 %) and *X. vasicola* pv. *holcicola* (90.92 %) (Table 2). The ANI calculation supports the conclusion that the three novel strains represent a novel species within the *Xanthomonas* group 2 clade, whose closest relatives are *X. oryzae* and *X. vasicola*.

PHENOTYPIC CHARACTERIZATION

Fatty acid profiles of the novel strains were generated following the protocol described by Roselló *et al*. [42]. Bacterial cultures were grown on trypticase soy broth agar (TSBA) at 28 °C for 24 h. Whole-cell fatty acid methyl esters (FAMEs) were obtained in four steps: saponification, methylation, extraction and sample cleaning [43], and they were analysed by gas chromatography (Hewlett-Packard 5890 gas chromatograph, Agilent Technologies). FAMEs extractions and chromatographic runs were performed twice for each bacterial strain.

Fatty acid profiles of the novel strains were typical of members of the genus *Xanthomonas*. They were very similar

Table 1. Percentage similarity between sequences of concatenated nucleotides of the *dnaK*, *fyuA*, *gyrB* and *rpoD* (3234 bases) of the novel isolates with other species of the genus *Xanthomonas*

	***X. prunicola* sp. nov.**		***X. prunicola* sp. nov.**
X. alfalfae	94.03±0.42	*X. fragariae*	90.78±0.57
X. arboricola	93.94±0.38	*X. gardneri*	93.06±0.48
X. axonopodis	93.67±0.39	*X. hortorum*	92.64±0.46
X. citri	93.86±0.41	*X. melonis*	92.33±0.50
X. bromi	93.97±0.46	*X. nasturtii*	93.60±0.50
X. campestris	90.82±0.54	*X. oryzae*	94.64±0.35
X. cassavae	92.38±0.43	*X. pisi*	94.54±0.41
X. codiaei	91.60±0.48	*X. populi*	90.99±0.56
X. cucurbitae	92.21±0.51	*X. vasicola*	94.98±0.34
X. cynarae	93.17±0.49	*X. vesicatoria*	93.17±0.47
X. euvesicatoria	94.14±0.41	*X. prunicola* sp. nov.	100
X. floridensis	92.80±0.40		

Table 2. ANIb values (percentages) obtained by pairwise comparisons between the novel isolates and all described species of the genus *Xanthomonas*

	***X. prunicola* sp. nov. CFBP 8353^T**	***X. prunicola* sp. nov. CFBP 8354**	***X. prunicola* sp. nov. CFBP 8355**
X. albilineans CFBP 2523^T	75.92	75.93	75.93
X. alfalfae CFBP 7686^T	89.40	89.41	89.41
X. arboricola CFBP 2528^T	86.69	86.69	86.70
X. axonopodis CFBP 4924^T	89.26	89.25	89.25
X. bromi CFBP 1976^T	89.30	89.31	89.30
X. campestris ATCC 33913^T	84.58	84.59	84.59
X. cassavae CFBP 4642^T	86.09	86.10	86.09
X. citri LMG 9322^T	89.40	89.40	89.40
X. codiaei CFBP 4690^T	85.87	85.87	85.88
X. cucurbitae CFBP 2542^T	85.06	85.07	85.06
X. cynarae CFBP 4188^T	86.31	86.32	86.31
X. dyei CFBP 7245^T	86.14	86.14	86.15
X. euvesicatoria LMG 27970^T	89.34	89.34	89.35
X. floridensis WHRI 8848 ^{T}	86.27	86.29	86.29
X. fragariae LMG 25863	85.96	85.98	85.98
X. fuscans CFBP 6165^T	89.45	89.45	89.45
X. gardneri ATCC 19865^T	86.39	86.41	86.40
X. hortorum CFBP 4925^T	86.31	86.30	86.30
X. hyacinthi CFBP 1156^T	78.19	78.20	78.20
X. melonis CFBP 4644^T	85.30	85.30	85.29
X. maliensis M97^T	81.35	81.35	81.36
X. nasturtii WHRI 8853 ^{T}	88.55	88.55	88.54
X. oryzae ATCC 35933^T	91.13	91.14	91.13
X. perforans CFBP 7293^T	89.52	89.53	89.52
X. pisi CFBP 4643^T	87.38	87.38	87.37
X. populi CFBP 1817^T	85.86	85.87	85.87
X. sacchari CFBP 4641^T	77.70	77.70	77.70
X. theicola CFBP 4691^T	78.15	78.17	78.16
X. translucens CFBP 2054^T	78.27	78.27	78.29
X. vasicola CFBP 2543^T	90.92	90.92	90.92
X. vesicatoria ATCC 35937^T	86.00	86.01	86.01
***X. prunicola* sp. nov. CFBP 8353^T**	100.00	99.99	99.99
***X. prunicola* sp. nov. CFBP 8354**	99.99	100.00	99.99
***X. prunicola* sp. nov. CFBP 8355**	99.97	99.97	100.00

to those obtained from ISPAVE B4 of *X. arboricola* pv. *pruni* and to the profiles determined for other species of the genus *Xanthomonas* in accordance with the descriptions of Saddler and Bradbury [44]. The predominant fatty acids were iso $C_{15:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso $C_{15:0}$ 2-OH), anteiso $C_{15:0}$ and $C_{16:0}$.

Phenotypic characterization of the novel strains was also performed using the miniaturized tests API 20 NE, API 50 CH (bioMérieux) and BIOLOG GN2 microplate system (Biolog). Bacterial strains were cultured on YPGA medium at 25 °C for 48 h. API 20 NE strips were inoculated with bacterial suspensions at OD_{600} 0.5 from 48 h old colonies from plates of YPGA medium and incubated at 25 °C for 48 h following the manufacturer's instructions. For API 50 CH strips, the bacterial suspensions were prepared in medium C [45] at OD_{600} 0.5 and the inoculated strips were incubated at 25 °C for 96 h. The tests were repeated twice and also performed with strains of different species of the genus *Xanthomonas* in order to compare them with the novel strains. Additionally, the metabolism of carbon compounds shown by the novel strains was evaluated using BIOLOG GN2 microplates, as described by other authors [46, 47], and compared with 24 strains of *X. arboricola* pathovars *corylina*, *juglandis*, *populi* and *pruni*, which were also included in the analysis because this species contains a pathovar affecting species of the genus *Prunus*. Briefly, bacterial strains were cultured on LB 2.0 % agar plates for 48 h at 27 °C. Subsequently, bacterial colonies were resuspended in PBS at OD_{600} 0.3 and 150 µl of each suspension were inoculated into the BIOLOG GN2 microplates. Absorbance was measured at 570 nm using a Labsystems Multiskan RC spectrophotometer (Fisher Scientific) after 48 h incubation at 27 °C. Three independent assays, including two microplates per strain and three reads per well, were performed. Means from the reads were calculated and compared to determine the level of substrate utilization relative to a negative control with no bacteria added. The utilization of the carbon compound was considered positive if the mean was at least 1.6 times higher than the negative control, and negative when the mean was 1.3 times lower [47]. Due to the presence of variable reactions among the evaluated strains, only the reactions with positive or negative results in the three independent assays were considered as valid. For the analysis, the results obtained were scored in a binary form for each compound as 0 (no utilization of the carbon source) and 1 (utilization of the carbon compound). Similarity among the strains was calculated by using the Jaccard coefficient and results were then subjected to UPGMA cluster analysis. Finally, the reliability of the tree thus obtained was determined using the Cophenetic Correlation Coefficient. All the analyses were computed on NTSYS 2.11T (Exeter Software) and graphically represented using Dendroscope software [48].

Phenotypic characteristics obtained with API 20 NE and API 50 CH tests revealed that the novel strains could be differentiated from other members of the genus *Xanthomonas* by the results obtained in nine tests (Table 3). Especially significant were the differences observed in the assimilation of mannitol and D-arabinose, which were positive in the novel strains and negative in the other strains of members of the genus *Xanthomonas*, with the exception of *X. oryzae* pv. *oryzae*, which showed a weak positive reaction with D-arabinose. Both phenotypic characters were useful for distinguishing the novel strains from the other species of the genus *Xanthomonas*. Analysis of the carbon metabolic profile using the BIOLOG GN2 microplate system revealed that the three novel strains presented a metabolic pattern that was different from that of the strains of *X. arboricola*, as represented in the dendogram obtained from the similarity analysis (Fig. 3). The three strains that are pathogenic to nectarine formed a homogeneous group that was distinct from all other *X. arboricola*, including those causal agents of bacterial spot disease on species of the genus *Prunus*. Fifteen carbon source compounds were utilized by the three novel strains, whereas 41 carbon sources were not used and 39 showed variable reactions and were therefore considered as not informative. According to this profile, the novel strains differed from the strains of *X. arboricola* in their ability to catabolize gentibiose, *cis*-aconitic acid and L-alaninamide.

All the results presented in this study indicated that the three isolates that are pathogenic to nectarine represent members of the genus *Xanthomonas*. The polyphasic approach used to identify these isolates included phenotypic methods (fatty acids profile, API 20 NE and API 50 CH tests and BIOLOG) and molecular analyses (sequence analysis of the 16S rRNA gene, MLSA and ANI). The results obtained confirmed that they formed a homogeneous group distinct from the other species of the genus *Xanthomonas*, and fulfilled the recommended criteria for the definition of a novel species [8]. It is therefore proposed that the novel strains be designated as representing a novel species of the genus *Xanthomonas*, and the name *Xanthomonas prunicola* sp. nov. is proposed.

DESCRIPTION OF *XANTHOMONAS PRUNICOLA* SP. NOV.

Xanthomonas prunicola (pru.ni'co.la. L. masc. or fem. suff. *-cola*….) (from L. n. *incola*), an inhabitant; N.L. fem. n. *prunicola* an inhabitant of *Prunus persica*).

Bacterial cells are Gram-stain-negative, non-spore-forming, strictly aerobic and straight rods. Colonies grown on YPGA medium for 48 h at 25 °C are circular, smooth, slightly convex, mucoid, 2–3 mm in diameter and produce yellow xanthomonadin pigment. They are catalase-positive, oxidase-negative, urease-negative, aesculin-positive and do not reduce nitrates. The predominant fatty acids are (in decreasing order): iso $C_{15:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso $C_{15:0}$ 2-OH), anteiso $C_{15:0}$, $C_{16:0}$, iso $C_{17:0}$, iso $C_{17:1}\omega9c$, iso $C_{11:0}$, $C_{12:0}$ 3-OH, iso $C_{13:0}$ 3-OH, $C_{15:0}$, iso $C_{16:0}$, $C_{14:0}$, iso $C_{11:0}$ 3-OH, $C_{17:1}\omega7c$, $C_{10:0}$, an unknown fatty acid of equivalent chain-length (ECL) 11 799, $C_{18:1}\omega9c$, $C_{18:1}\omega7c$ and, as a minor component, anteiso $C_{17:0}$. In the API 20NE system

Table 3. Phenotypic characteristics that differentiate three novel strains isolated from nectarine from reference strains of other members of the genus *Xanthomonas*

Taxa: 1, *X. prunicola* sp. nov.; 2, *X. arboricola* pv. *juglandis* CFBP 2528^{T}; 3, *X. arboricola* pv. *populi* CFBP 3123^{PT}; 4, *X. arboricola* pv. *pruni* CFBP 3894^{PT}; 5, *X. campestris* pv. *campestris* CFBP 5241^{T}; 6, *X. cynarae* CFBP 4188^{T}; 7, *X. hortorum* pv. *hederae* CFBP 4925^{T}; 8, *X. oryzae* pv. *oryzae* CFBP 2532^{T}; 9, *X. vasicola* pv. *holcicola* CFBP 2543^{T}; +, positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9
In API 20NE:									
Gelatin	+	+	+	+	+	+	+	−	−
Mannitol	+	−	−	−	−	−	−	−	−
Maltose	+	+	+	−	−	−	−	−	−
In API 50CH:									
D-arabinose	+	−	−	−	−	−	−	w	−
L-arabinose	−	−	−	−	−	+	−	−	−
N-acetylglucosamine	−	−	w	w	−	w	−	+	w
Salicin	−	−	+	−	−	−	−	−	−
D-fucose	+	+	w	w	w	+	−	−	−
L-fucose	+	−	w	−	+	w	−	−	w

(bioMérieux), positive for β-glucosidase and β-galactosidase activity, gelatin and the assimilation of glucose, mannose, mannitol, *N*-acetyl-glucosamine, maltose, malic acid and tri-sodium citrate, but negative for the other tests of the strip. In the API 50CH (bioMérieux), only D-arabinose, aesculin, D-fucose and L-fucose are utilized. Using BIOLOG GN2 microplates, the strains show metabolic activity on the following carbon sources: cellobiose, D-fructose, gentibiose, α-D-glucose, D-mannose, D-psicose, sucrose, trehalose, *cis*-aconitic acid, α-ketoglutaric-acid, succinic acid, bromosuccinic acid,

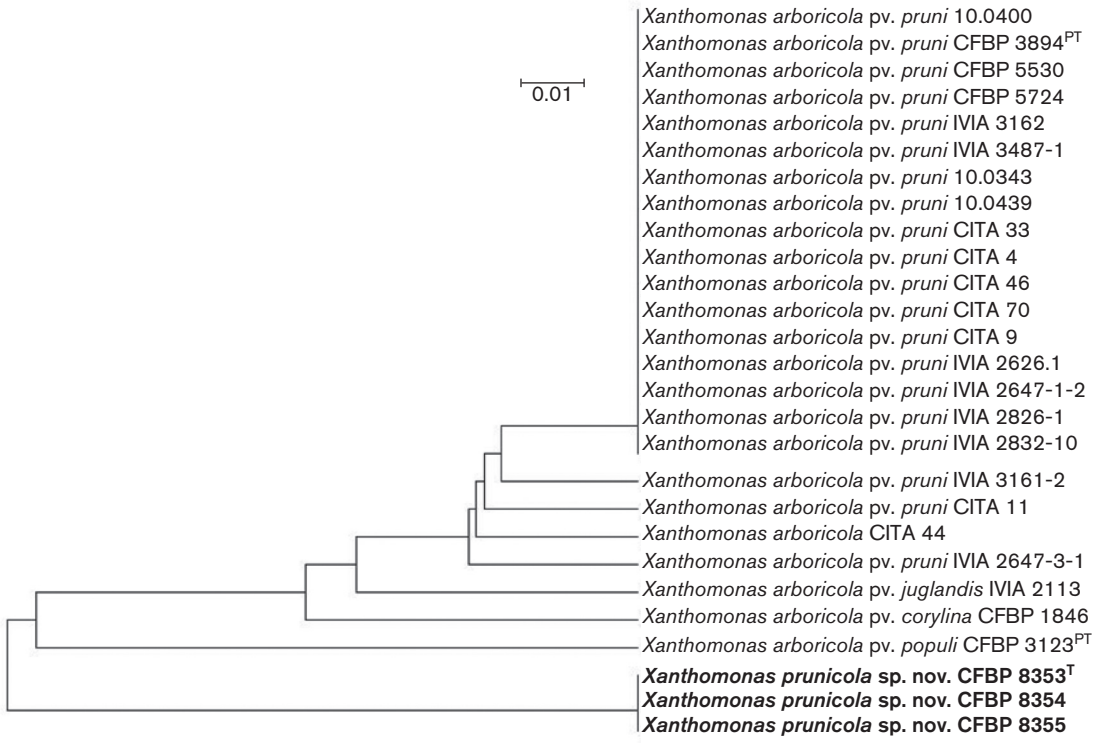

Fig. 3. Comparative cluster analysis of the carbon compound utilization profile of novel isolates of members of the genus *Xanthomonas* and those of *X. arboricola* according to the Biolog GN2 microplate system. Cluster analysis was performed based on 57 informative substrates. Data were computed using the UPGMA model. Reliability of the tree was determined by the Cophenetic Correlation Coefficient (r=0.988). Novel isolates are indicated by bold type.

L-alaninamide, L-glutamic acid and glycyl L-aspartic acid. The strains lack metabolic activity on: α-cyclodextrin, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, i-erythritol, *myo*-inositol, D-mannitol, D-melobiose, raffinose, L-rhamnose, D-sorbitol, acetic acid, citric acid, formic acid, D-galactonic-acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, γ-hydroxybutyric acid, *p*-hydroxy phenylacetic acid, itaconic acid, α-ketovaleric acid, sebacic acid, glucuronamide, L-asparagine, L-histidine, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, γ-aminobutyric acid, inosine, uridine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2–3 butanediol, DL-α-glycerol phosphate, α-D-glucose and D-glucose 6-phosphate.

PCR analyses yield negative results using the *X. arboricola* pv. *pruni* primers [31, 32].

Strains are clearly differentiated from all other species of the genus *Xanthomonas* by MLSA based on a concatenated sequence of the *dnaK*, *fyuA*, *gyrB* and *rpoD* genes and ANI values obtained by whole-genome comparisons.

Strains are pathogenic to *P. persica*, producing necrotic spots after inoculation on detached leaves and whole plants. Additionally, they elicit a hypersensitivity reaction when inoculated in tobacco and tomato leaves.

The type strain is IVIA 3287.1 (=CFBP 8353^T=CECT 9404^T).

Funding information
This work was supported by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) research programme (project RTA2011-00140-C03-00). P. López-Soriano was supported by a fellowship provided by the INIA (FPI-INIA).

Acknowledgements
We especially thank Clara Morente, Carmina Salcedo and María Teresa Gorris for their excellent technical work, Francesc Garcia for FAME analyses and Marco Scortichini for kindly sending the *X. arboricola* pv. *pruni* reference strain. The authors thank the ANAN platform (Angers, France) for providing facilities for genome sequencing, and Martial Briand for IT support for genome analysis.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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