Pathogenic, phenotypic and molecular characterisation of *Xanthomonas nasturtii* sp. nov. and *Xanthomonas floridensis* sp. nov., new species of *Xanthomonas* associated with watercress production in Florida

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ABSTRACT

We describe two new species of the genus *Xanthomonas*, represented by yellow mucoid bacterial strains isolated from diseased leaves of watercress (*Nasturtium officinale*) produced in Florida, USA. One strain was pathogenic on watercress, but not in other species including a range of brassicas; other strains were not pathogenic in any of the tested plants. Data from Biolog carbon source utilization tests and nucleotide sequence data from 16S and *gyrB* loci suggested that both pathogenic and non-pathogenic strains were related to, yet distinct from, previously described *Xanthomonas* species. Multilocus sequence analysis and whole genome-wide comparisons of average nucleotide identity (ANI) of genomes of two strains from watercress showed that these are distinct and share less than 95% ANI with all other known species; the non-pathogenic strain WHRI 8848 is close to *X. cassavae* (ANI of 93.72%) whilst the pathogenic strain WHRI 8853 is close to a large clade of species that includes *X. vesicatoria* (ANI ≤90.25%). Based on these results, we propose that both strains represent new *Xanthomonas* species named *X. floridensis* sp. nov. (type strain WHRI 8848=ATCC TSD-60=ICMP 21312=LMG 29665=NCPPB 4601) and *X. nasturtii* sp. nov. (type strain WHRI 8853=NCPPB 4600=LMG 29666=ICMP 21313=ATCC TSD-61), respectively. The presence of non-pathogenic *Xanthomonas* strains in watercress and their interaction with pathogenic strains needs to be further investigated. Although the importance of the new pathogenic species is yet to be determined, the bacterial disease that it causes constitutes a threat to watercress production and its distribution should be monitored.

Keywords: *Xanthomonas*; black rot; watercress; pathogenicity; new species; *Brassicaceae*

The GenBank accession numbers for the partial sequences of two strains from watercress (WHRI 8848 and 8853) are KX518636 and KX518637 for 16S and KX523290 and KX523291 for *gyrB*. The GenBank accession numbers for the genome sequences are LXNG00000000.1 and LYMI00000000.1 for WHRI 8848 and WHRI 8853, respectively.

Six supplementary tables and four supplementary figures are available.

The genus *Xanthomonas*, first described by Dowson in 1939, comprises at least 28 species (Bull *et al.*, 2010, Bull *et al.*, 2012). The classification of these species has had multiple changes with inclusion of new strains and re-classification of groups of strains (Bull *et al.*, 2010, Constantin *et al.*, 2016, Jacques *et al.*, 2016, Parkinson *et al.*, 2009, Vauterin *et al.*, 2000). In addition, there are a number of strains still included as different pathovars of several species (e.g. pathovars of *X. campestris*); some of these strains/pathovars will most likely be reclassified in the future (Bull *et al.*, 2010).

Bacteria from this genus are generally plant-associated and include many economically important pathogens (Vauterin *et al.*, 1995). Until this study, *Xanthomonas campestris* has been considered the only *Xanthomonas* species that causes diseases in *Brassicaceae* crops, weeds and ornamental plants (Vauterin *et al.*, 1995, Vicente *et al.*, 2006, Vicente & Holub, 2013). Morphological characteristics, classical phenotypic tests, fatty acid methyl esters analyses, serology based assays and DNA-DNA hybridization have been used to characterise strains and classify *Xanthomonas* species. Molecular PCR-based methods are now routinely used for the identification of diseases caused by *Xanthomonas* spp. with 16S ribosomal DNA sequences being used for identification at the genus level (Hauben *et al.*, 1997) and conserved genes including *gyrB* at species or sub-species level (Parkinson *et al.*, 2007, Parkinson *et al.*, 2009). Multilocus sequence analysis is an effective method to discriminate species and some pathovars of *Xanthomonas* (Almeida *et al.*, 2010, Young *et al.*, 2008). Whole genomes of several hundreds of strains of *Xanthomonas* spp. have been sequenced revealing insights into infection biology, evolutionary processes and phylogenetic relationships (Denance *et al.*, 2016, Jacques *et al.*, 2016, Ryan *et al.*, 2011). In particular, this approach has begun to reveal putative novel *Xanthomonas* species (Aritua *et al.*, 2015, Jacobs *et al.*, 2015, Pieretti *et al.*, 2015, Triplett *et al.*, 2015). Nevertheless, inoculation of susceptible plant hosts remains indispensable for confirmation of pathogenicity of strains involved in plant disease.

Watercress (*Nasturtium officinale* R. Br.) is a rapidly growing aquatic or semi-aquatic perennial member of the *Brassicaceae* and is one of the oldest salad crops that has been harvested from the wild and from cultivation in many regions around the world. Watercress is considered a super-food, rich in nutrients and with anti-carcinogenic properties (Fogarty *et al.*, 2013, Payne *et al.*, 2005). Watercress is a

rapid coloniser of waterways and wetlands, and is an invasive or potentially invasive plant in several countries (CABI, 2017). Commercial production started in England at the beginning of the 19th century and was afterwards introduced in the USA and other parts of the world (CABI, 2017). The crop is either produced in outdoor flooded beds with slow flowing water, or in greenhouse hydroponic systems. In summer, the waterbeds are emptied and cleaned between crop cycles, whilst in winter, the crop remains in the beds for longer periods as plants grow slower, and these conditions can be favourable for disease development and spread.

Disease problems in watercress are commonly caused by fungi such as *Cercospora nasturtii* causing leaf spot and *Spongospora subterranea* causing crook-root (McHugh *et al.*, 1987, Strandberg & Tucker, 1968), by viruses such as watercress yellow spot virus (Walsh & Phelps, 1991) and by the phytoplasma Aster Yellows (McHugh & Constantinides, 2004). A bacterial disease referred to as black rot, caused by *Xanthomonas campestris*, has also been cited to affect watercress (CABI, 2017). In Hawaii, black rot has been referred to as a common disease of watercress in all islands and sites where it is grown, especially in beds protected from wind and when humidity is high due to rain or overhead irrigation; however the causal agent has not been identified and studied (McHugh & Constantinides, 2004). This disease was not included in a list of diseases of watercress in Florida (Strandberg & Tucker, 1968) and is rarely mentioned in UK lists.

Symptoms that could be attributed to a bacterial disease were observed on plants grown commercially in flooded beds in Florida. For the current study, leaves of watercress produced in Florida were received in January 2014 and bacteria were isolated from chlorotic yellow and dark lesions (Fig. S1) by excising and macerating small fragments (3mm^2) of tissue from the edge of lesions in sterile water (300µl) and plating loopfuls of the suspension in King's B medium (King *et al.*, 1954) and yeast dextrose calcium carbonate agar (YDC) (Stolp & Starr, 1964). Plates were incubated at 28°C for 48-72 h and observed for typical *Xanthomonas* (yellow, mucoid) and/or *Pseudomonas* colonies (fluorescent, cream-yellow). Single colonies were sub-cultured in KB and YDC plates and pure isolates including five *Xanthomonas*-like (non-fluorescent, yellow-mucoid in KB and YDC) and four *Pseudomonas*-like isolates (blue fluorescent and cream-yellow on KB plates) were stored at -76°C in cryovials containing beads and nutrient broth with 15% glycerol according to Feltham *et al.*

(1978) (Table 1). Colony morphology and pigment production of two isolates (WHRI 8848 and 8853) were observed in KB and YDC plates over a period of ten days; one of these isolates (WHRI 8853) was distinct from the others as it produced a brown pigment in KB plates (Fig. 1). Whole cells of strains WHRI 8848 and 8853 were negatively stained and observed using a Field Emission Electron microscope; cells were rod shaped with a single polar flagellum typical of *Xanthomonas* (Fig. 2).

All bacterial isolates obtained from watercress (Table 1) were initially tested for pathogenicity on watercress and Savoy cabbage cv. Wirosa F_1 plants; control isolates of *X. campestris* pathovars known to infect *Brassicaceae* and *X. axonopodis* pv. *phaseoli* that infect beans, were included. The *Xanthomonas* isolates from watercress were subsequently tested on three watercress accessions and a wide range of potential hosts including brassicas, radish, ornamental crucifers, tomato and beans (Table S1). In addition, three watercress accessions and brassica and bean lines were tested with representative strains of *X. campestris* pathovars known to cause disease in *Brassicaceae* spp. and *X. axonopodis* from bean (Table S2). Bacterial isolates were recovered from cultures stored at -76°C and grown on KB agar plates at 28°C for 48 h. Four-week-old plants were inoculated by piercing at least three young leaves in multiple points including the middle and some secondary veins, using a pin charged with bacterial growth diluted in a drop of distilled water (method adapted from Vicente et al. (2006)). For beans, the first two trifoliate leaves were inoculated in several points. Inoculation experiments were repeated at least once. Inoculations of watercress with three *X. campestris* pv. *raphani* isolates were also performed using a spray inoculation method as described in Vicente *et al.* (2006). The presence of symptoms was recorded one and two weeks after inoculation for plants inoculated with *X. campestris* pv. *raphani* and two and three weeks after inoculation for all others isolates according to the methods previously developed for inoculation of brassicas (Vicente *et al.*, 2001, Vicente *et al.*, 2006).

Only one isolate from watercress (WHRI 8853) produced disease symptoms in watercress (Fig. 3) and none of the isolates produced symptoms in Wirosa F_1 plants (Table 1). Re-isolations were made from a leaf of watercress with black rot symptoms following the method described above and isolates with the same morphological characteristics of WHRI 8853 were obtained and the pathogenicity was confirmed in watercress. None of the *Xanthomonas* strains from watercress produced symptoms in

a range of potential host species; WHRI 8853 and 8930A (a re-isolation of WHRI 8853) were pathogenic in the three watercress accessions tested, but not on any brassica and other hosts (Table S1). Symptoms caused by isolate WHRI 8853 after inoculation of watercress are similar to black rot symptoms caused in brassicas by *X. campestris* pv. *campestris* (Fig. 3).

Watercress was resistant or moderately resistant to standard isolates of *X. campestris* pv. *campestris* and *X. axonopodis* pv. *phaseoli,* and generally susceptible to *X. campestris* pv. *raphani,* although there were some variable reactions (Fig. S2, Table S2). This indicates that new strains such as WHRI 8853 have a distinct host range and might be more aggressive and adapted to watercress than other known pathogenic species/pathovars of *Xanthomonas*.

Biochemical tests including 71 carbon source and 23 chemical sensitivity assays were performed using the Biolog GEN III Microplates™ (Biolog Inc., Hayward, CA, USA) to profile six strains from watercress (WHRI 8844, 8846A, 8848, 8851, 8853 and 8930A) and representative strains of *X. campestris* pv. *campestris* and *X. axonopodis* pv. *phaseoli*. One microplate was done per strain except for strains WHRI 8848 and 8853 that were repeated at different dates. Isolates were grown on Biolog Universal Growth Agar (BUG™) medium (Biolog Inc., Hayward, CA) for 24h at 28°C. Bacterial growth was removed from plates with sterile swabs and suspended in inoculating fluid IF-A (Biolog Inc., Hayward, CA) according to manufacturers protocol A. Transmittance was checked on a spectrophotometer (Jenway 6315) at 600nm. Microplate wells were filled with 100 µl of bacterial suspension and the plates were incubated at 29±1°C. Plates were photographed and visually recorded for purple colour reaction at 18, 22, 24 and 48 hours. The results obtained at 22h were compared with the Biolog database as recommended by the manufacturer. The watercress isolates were identified as *X. campestris* or *X. axonopodis* (Table S3). The isolate WHRI 1279A from cabbage was confirmed as *X. campestris* pv. *campestris* and the isolate WHRI 1925C from bean was identified as *X. hortorum* pv. *carotae*. Although at the genus level these identifications were accurate, the small number of *Xanthomonas* spp. (under 10 species) currently included in the database limits the identification at the species level. It was also noticed that at 22h a number of reactions were still developing and therefore the results were not very consistent.

Vauterin *et al.* (1995) found that reactions were better developed and more reliable after 48h and therefore used this incubation period for comparison of *Xanthomonas* groups. Selected results of Biolog GEN III microplate assays obtained at 48h are presented in Table 2 and the complete results are presented in a supplementary table (S4). Four tests (propionic acid, D-saccharic acid, α-ketobutyric acid and D-raffinose) were consistent and allowed the differentiation between the watercress isolates and 21 other *Xanthomonas* species in comparison with results of Vauterin *et al.* (1995), Tréobal *et al.* (2000) and Triplett *et al.* (2015) (Table 2). Several tests also differentiate the watercress isolates from isolates of nine species included in a study by Constantin *et al.* (2016) (Table S5). In addition, D-saccharic acid, mucic acid, gentiobiose and D-salicin clearly differentiated the two groups of watercress isolates; D-maltose and D-mannitol differentiated the watercress isolates from the two control isolates used in this study (*X. campestris* WHRI 1279A and *X. axonopodis* WHRI 1925C) and gelatin differentiated between the two control isolates (Tables S3 and S4). Previous studies have shown that groups of strains of each species are often heterogeneous and some species might not be clearly differentiated using Biolog (Constantin *et al.*, 2016, Vauterin *et al.*, 1995). Nevertheless, Biolog was shown to be a sensitive technique that includes tests that can differentiate between the two groups of *Xanthomonas* isolates from watercress and between these groups and at least 23 other species previously characterised (listed in supplementary Table S6).

For molecular analyses, DNA extraction was performed with the Qiagen DNeasy tissue extraction kit (Qiagen Ltd., West Sussex, UK) following the method described previously (Vicente *et al.*, 2006) and the manufacturer's protocol for extraction of DNA from bacteria and animal tissues. The 16S universal primers 27F (5'- AGAGTTTGATCMTGGCTCAG -3') and 1492R (5' – GGTTACCTTGTTACGACTT – 3') (Jiang *et al.*, 2006) were used to amplify a partial sequence of all isolates. The *gyrB* forward (5' – ACGAGTACAACCCGGCAA – 3') and reverse (5' – CCCATCARGGTGCTGAAGAT – 3') primers (Young *et al.*, 2008) were used for PCR amplification from all isolates confirmed as *Xanthomonas* according to 16S sequencing. All primers were synthesised by Sigma-Aldrich (Dorset, UK) and diluted to 10 µM. Positive controls (listed in Table 1) and blanks with water used as template were included in all PCR runs. PCRs for amplification of 16S were conducted in 25

µl reaction volume containing 0.5µl of template DNA solution (approximately 25 ng of bacterial DNA), 5µl of 5x HiFi Reaction buffer, 2.5 µl of dNTP mix at 10 mM, 1 µl of each forward and reverse primers at 10 µM and 0.5 µl of Velocity High fidelity DNA polymerase (Bioline). The reactions were done in a GeneAmp PCR System 9700 (Applied BioSystems) thermal cycler with the conditions: denaturation 98°C for 3 min, 35 cycles of denaturation at 98°C for 30s, annealing at 56°C for 30s and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min. PCRs for amplification of *gyrB* were conducted in 25 µl reaction volume containing 0.5µl of template DNA solution (approximately 25 ng of bacterial DNA), 2.5µl of 10X buffer with $MgCl_2$, 2.5 µl of dNTP mix at 10 mM, 1 µl of each forward and reverse primers at 10 µM and 0.25 µl of Long PCR Enzyme mix (Fermentas). The reactions were done in a GeneAmp PCR System 9700 (Applied BioSystems) thermal cycler. The conditions for *gyrB* amplification were the same as 16S except that the denaturation temperature was 94°C and the extension was 68°C. Purity of the PCR products was checked by running 4 µl reaction mixture on a 1% agarose gel stained with GelRed™ nucleic acid gel stain (Biotium Inc. Hayward, CA, USA) by electrophoresis for 1 hour at 120V and the gels were visualised and photographed on an UV transilluminator and a G:Box gel imaging system (Syngene, Cambridge, UK). PCR products were purified using a Bioline Isolate II PCR and Gel Kit (Bioline Reagents Limited, London, UK) and sent for Sanger sequencing to GATC (GATC Biotech AG, Cologne, Germany). Sequence analysis was performed using the DNASTAR software package (DNASTAR, Inc., Madison, WI USA). Sequences were compared with sequences available in the National Center for Biotechnology Information (NCBI) databases using Basic Local Alignment Search Tool (BLAST).

Partial sequences of 16S were obtained for *Xanthomonas* and *Pseudomonas*-like isolates. BLAST comparisons of sequences approximately 1300bp long, showed that six non-fluorescent isolates (WHRI 8844, 8846A, 8848, 8851, 8853 and its re-isolation, 8930A) were most likely *Xanthomonas* spp.; four fluorescent isolates (WHRI 8845, 8850, 8854 and 8855) were *Pseudomonas* spp., possibly *P. cichorii*, *P. fluorescens* and *P. koreensis* (Table 1) although identification of these organisms at the species level is not reliable using only 16S sequences as stated in previous studies (Hauben *et al.*, 1997). Partial *gyrB* sequences (ca. 820 bp) were obtained for all six *Xanthomona*s isolates. BLASTN searches against the NCBI Nucleotide database that

includes *gyrB* sequences of all described species of *Xanthomonas* (Table S6), revealed that *gyrB* sequences from four isolates (WHRI 8844, 8846A, 8848, 8851) shared 97% identity with *X. cassavae*, 95% with *X. codiaei* and 93% with *X. cucurbitae*, whilst WHRI 8853 and 8930A *gyrB* sequences were only 93% identical to sequences from a range of species including *X. axonopodis*, *X. citri*, *X. fuscans*, *X. perforans*, *X. vasicola* and *X. oryzae* (Table 1). Partial sequences of two *Xanthomonas* strains from watercress (WHRI 8848 and 8853) were deposited in GenBank under accession numbers KX518636 and KX518637 for 16S and KX523290 and KX523291 for *gyrB.*

For whole genome sequencing, genomic libraries of the *Xanthomonas* strains WHRI 8848 and 8853 from watercress were prepared at the Genomic Centre, School of Life Sciences, University of Warwick, using the Illumina Nextera XTDNA kit (Illumina, San Diego, USA) following the manufacturer's instructions. The average fragment lengths were 600-700bp, but covered a range from 200bp to over 1000bp. AMPure XP beads (Beckman Coulter, High Wycombe, UK) were used for purification after amplification. The libraries were then sequenced using a Rapid v2 500-cycle kit topped-up to run 600 cycles on a 2x300bp Rapid Run on a HiSeq 2500 sequencing system at Illumina Cambridge Ltd (Essex, UK). The quality of the sequence data was checked using FastQC (Andrews, 2016). Poor-quality and adaptor-containing reads were filtered and trimmed using TrimGalore (Krueger, 2016). Genomes were assembled using SPAdes v. 3.6.2 (Bankevich *et al.*, 2012) and scaffolded using SSPACE v. 3.0 (Boetzer *et al.*, 2010). The quality of assemblies and summary statistics were calculated with Quast (Gurevich *et al.*, 2013). Genome assemblies were annotated using Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) at the NCBI. Genome sequence assemblies were aligned and compared using the *dnadiff* script, which is a wrapper script for the Nucmer tool in MUMmer (Delcher *et al.*, 2002).

Multi-locus sequence analysis (MLSA) based on four genes of 21 species (Table S6) was consistent with the 16S rDNA and *gyrB* sequence results (Fig. 4). This placed strain WHRI 8848 as closely related to, yet distinct from *X. cassavae*, the causative agent of bacterial necrosis in cassava. Strain WHRI 8853 was not closely related to any known species, but fell within a well-supported clade within the genus *Xanthomonas* that includes a number of species (e.g. *X. oryzae*, *X. vasicola* and *X. vesicatoria*).

We performed further genome-wide comparisons of average nucleotide identity (ANI) between the genomes of strains WHRI 8848 and 8853 and other available genome sequences from related species (Table S6) using both *dnadiff* and JSpecies (Richter & Rosselló-Móra, 2009). ANI values between members of a single species usually exceed 95% (Richter & Rosselló-Móra, 2009). The genome sequences of WHRI 8848 and 8853 share less than 95% ANI with previously sequenced representatives of *Xanthomonas* species, and therefore do not fall within any of these species (Fig. S3). Consistent with the MLSA, the genome sharing the highest ANI with WHRI 8848 is that of a strain of *X. cassavae*, with which it shares only 93.72%. Again consistent with the MLSA, WHRI 8853 shares no more than 90.25% with any previously sequenced *Xanthomonas* genome. Therefore, the combined molecular evidence from MLSA and ANI indicates that strains WHRI 8848 and 8853 each represent members of previously undescribed species.

Strain WHRI 8853 encodes a predicted non-flagellar type-three secretion system (T3SS) as well as several T3SS effector proteins, which is consistent with the ability of *Xanthomonas* pathogens to infect and cause disease in plants (Fig. S4). A search using T346Hunter (Martínez-García *et al.*, 2015) identified a 66.8-kb contig (GenBank: LYMI01000021.1) containing a cluster of genes encoding a complete T3SS apparatus including effectors proteins HpaA, XopAE, XopF1 and XopZ. Immediately adjacent to the T3SS gene cluster is a gene encoding the effector AvrBs2. This juxtaposition of *avrBs2* and the T3SS apparatus genes is also observed in *X.* sp. Nyagatare (Aritua *et al.*, 2015), but not in other sequenced *Xanthomonas* genomes. Also encoded in the WHRI 8853 genome are sequences encoding full-length homologues of T3SS effector proteins XopAD, XopAG, XopD, XopJ3, XopN and XopX and the avirulence protein AvrXccA1. Furthermore, there is a homologue of a transcription activator-like (TAL) effector gene, though it is split across two contigs (GenBank: LYMI01000075.1 and LYMI01000085.1).

In contrast, WHRI 8848 does not contain genes encoding a non-flagellar T3SS or any recognisable T3SS effector proteins, though it does contain a homologue of the avirulence gene *avrXccA1*. Although the T3SS is usually considered to be a key pathogenicity determinant and the majority of previously studied *Xanthomonas* strains encode a T3SS and accompanying effectors, a few *Xanthomonas* strains have been isolated that lack a T3SS (Cesbron *et al.*, 2015, Essakhi *et al.*, 2015, Jacobs *et al.*,

2015, Triplett *et al.*, 2015). In common with WHRI 8848, these previously reported non-T3SS xanthomonads are apparently non-pathogenic. These studies suggest that significant diversity of non-pathogenic and T3SS-free xanthomonads has yet to be discovered. Most research on *Xanthomonas* has been motivated by its importance in disease resulting in a bias towards T3SS-bearing pathogens. However, microbiome studies are beginning to reveal members of the *Xanthomonas* genus as a significant constituent of microbial communities associated with plants that show no signs of disease (de Campos *et al.*, 2013, Takahashi *et al.*, 2011, Vorholt, 2012). The interactions between pathogenic and non-pathogenic bacteria is not clearly understood as highlighted in a study of two contrasting groups of *Xanthomonas* strains from banana (Adriko *et al.*, 2016). The presence of non-pathogenic *Xanthomonas* in diseased leaves and the role of *Pseudomonas* spp. in pathogenicity in watercress should be further investigated.

Based on pathogenicity, Biolog phenotypic testing and sequencing data, we propose that both non-pathogenic and pathogenic sequenced strains are new *Xanthomonas* species, *X. floridensis* and *X. nasturtii,* respectively. This is the first characterisation of *Xanthomonas* isolates obtained from watercress and the first study describing the pathogen responsible for a vascular disease that could be called black rot or bacterial wilt of watercress. Koch's postulates were satisfied for the pathogenic isolate as the microorganisms that were re-isolated from a diseased watercress leaf (WHRI 8930A) were confirmed as being similar to isolate WHRI 8853 in pathogenicity tests, Biolog assays and sequencing of *gyrB*. Although the importance of this pathogen is still unknown, this disease should be considered a threat to watercress production. As a fast growing crop that is produced mainly to be consumed as fresh salad, the disease control options and pesticides available for watercress are very limited. The control measures recommended in Hawaii included reducing humidity levels by limiting overhead irrigation and increasing air circulation, and use of copper hydroxide (McHugh & Constantinides, 2004). There is little genetic variation in commercial watercress varieties (Sheridan *et al.*, 2001) although there is more variation in other accessions (Payne *et al.*, 2005), so it might be possible to select resistant watercress accessions by screening accessions with a strain of *X. nasturtii*. It is also important to test seed lots for *Xanthomonas* spp. to determine whether this pathogen is present as it is assumed that this pathogen is seed-borne, and

crops in different locations should be regularly monitored to access the spread of this pathogen.

Description of *Xanthomonas floridensis* sp. nov.

Xanthomonas floridensis (flo.ri.den'sis N.L. fem. adj. floridensis from of or belonging to Florida, the state in the United States from which the strains were isolated).

Strains of this species are not known to be pathogenic. Bacterial cells are Gram-negative straight rods with a single polar flagellum. Colonies grown on yeast dextrose calcium carbonate agar for two days at 28°C are yellow, circular (approximately 2mm in diameter), smooth, mucoid, glistening and slightly convex. Colonies grown on King's B medium for two days are circular, non-fluorescent, cream yellow. As determined by Biolog GENIII microplate tests, *Xanthomonas floridensis*, gives metabolic activity on 29 carbon substrates including D-fructose, α-D-glucose, D-mannose (characteristic of the genus), propionic acid, D-maltose, D-mannitol, gelatin, D-saccharic acid and gentiobiose. It lacks metabolic activity on 27 carbon substrates including D-serine, γ–aminobutyric acid (characteristic of the genus), D-raffinose and L-rhamnose (full list included in Table S4). The assembled genome of the type strain (available at http://www.ncbi.nlm.nih.gov/nuccore/LXNG00000000) is 5,254,357 bp and the DNA G + C content is 65.14%. The type strain is WHRI 8848 (=ATCC TSD-60=ICMP 21312=LMG 29665=NCPPB 4601) isolated from leaves of watercress grown in Florida.

Description of *Xanthomonas nasturtii* sp. nov.

Xanthomonas nasturtii (nas.tur'ti.i. N.L. gen. neut. n. nasturtii of Nasturtium from the genus name of watercress, *Nasturtium officinale* R. Br.)

Typical symptoms of the disease in watercress resemble back rot of brassicas (caused by *X. campestris* pv. *campestris*) and might be named black rot or bacterial wilt of watercress. Symptoms consist of yellow V-shape lesions frequently with darker veins and darker, necrotic centre. Bacterial cells are Gram-negative straight rods with a single polar flagellum. Colonies grown on yeast dextrose calcium carbonate agar for two days at 28°C are yellow, circular (approximately 2 mm in

diameter), smooth, mucoid, glistening and slightly convex. Colonies grown on King's B medium for two days are circular, non-fluorescent, cream yellow turning to darker yellow and at 3-4 days the medium becomes brown due to the production of pigments (fuscous). As determined by Biolog GENIII microplate tests, *X. nasturtii*, gives metabolic activity on 23 carbon substrates including D-fructose, α-D-glucose, D-mannose (characteristic of the genus), propionic acid, D-maltose, D-mannitol, and gelatin. It lacks metabolic activity on 36 carbon substrates including D-serine, γ–aminobutyric acid (characteristic of the genus), D-raffinose, L-rhamnose, D-saccharic acid, gentiobiose and formic acid (full list included in Table S4). The assembled genome of the type strain (available at http://www.ncbi.nlm.nih.gov/nuccore/LYMI00000000) is 4,873,771 bp and the DNA G + C content is 63.72%. The type strain is WHRI 8853 (=ATCC TSD-61=ICMP 21313=LMG 29666=NCPPB 4600) isolated from leaves of watercress grown in Florida.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Table 1. Bacterial isolates obtained in the current study from watercress samples from Florida, US, and isolates used as controls in pathogenicity tests and 16S and *gyrB* PCR identification.

WHRI number (race type)	Origin (year)	Host	Morphology[*]	Pathogenicity[**] on		16S / *gyrB* identification (example of top hit species[†])
				Watercress	Wirosa F_1	
Xanthomonas isolates from watercress						
8844, 8846A, 8848, 8851	Florida, US (2014)	*Nasturtium officinale*	NF, T	-	-	*Xanthomonas* sp. (*X. cassavae*) [††]
8853	Florida, US (2014)	*Nasturtium officinale*	NF, Fuscous	+	-	*Xanthomonas* sp. (*X. axonopodis*) [††]
8930A	8853 re-isolation (2015)	*Nasturtium officinale*	NF, Fuscous	+	-	*Xanthomonas* sp. (*X. axonopodis*)
Pseudomonas isolates from watercress						
8845	Florida, US (2014)	*Nasturtium officinale*	F	-	-	*Pseudomonas* sp. (*P. cichorii*) [§]
8850	Florida, US (2014)	*Nasturtium officinale*	F	-	-	*Pseudomonas* sp. (*P. fluorescens*) [§]
8854, 8855	Florida, US (2014)	*Nasturtium officinale*	F	-	-	*Pseudomonas* sp. (*P. koreensis*) [§]
Xanthomonas campestris pv. *campestris* controls[§§]						
3811 (race 1)	US	*Brassica oleracea*	NF, T	- / (+)	+	*Xanthomonas* sp. (*X. campestris*)
1279A (race 4)	Cornwall, UK (1984)	*Brassica oleracea*	NF, T	- / (+)	+	*Xanthomonas* sp. (*X. campestris*)
6181 (race 6)	Sardoal, Portugal (1996)	*Brassica rapa*	NF, T	- / (+)	+	*Xanthomonas* sp. (*X. campestris*)
Xanthomonas campestris pv. *raphani* controls[§§]						
6490 (race 1)	France (1995)	*Brassica oleracea*	NF, T	- / Ls	Ls	*Xanthomonas* sp. (*X. campestris*)
8305 (race 2)	Oklahoma, US (1995)	*Brassica rapa*	NF, T	- / Ls	Ls	*Xanthomonas* sp. (*X. campestris*)
6519 (race 3)	Shizuoka, Japan (1985)	*Raphanus sativus*	NF, T	- / Ls	Ls	*Xanthomonas* sp. (*X. campestris*)
Xanthomonas campestris pv. *incanae* control[§§]						
6378	US (1949)	*Matthiola incana*	NF, T	-	-	*Xanthomonas* sp. (*X. campestris*)
Xanthomonas axonopodis pv. *phaseoli* controls						
924A	UK (1976)	*Phaseolus vulgaris*	NF, Fuscous	-	-	*Xanthomonas* sp. (*X. axonopodis*)
1925C	Rwanda (1987)	*Phaseolus vulgaris*	NF, Fuscous	-	-	*Xanthomonas* sp. (*X. axonopodis*)
3342A[#]	Uganda (1993)	*Phaseolus vulgaris*	NF, T	-	-	*Xanthomonas* sp. (*X. axonopodis*)

[*] Observed in King's B medium. NF= non-fluorescent, F = Fluorescent, T = typical pigmentation, Fuscous = brown diffuse melanin pigment

[**] Four week-old-plants were grown, inoculated and recorded following methods previously developed (Vicente *et al.*, 2001, Vicente *et al.*, 2006). Black rot or vascular symptoms: +, compatible interaction (susceptible host); -, incompatible interaction (resistant host); (+) weakly pathogenic; -/(+), variable result within accession. Ls, leaf spot symptoms, necrosis around inoculation points and sunken lesions in middle veins. Watercress from Thompson & Morgan, UK and Savoy cabbage cv. Wirosa F_1 from Bejo Zaden NV.

[†] Based on BLAST comparisons of 16S sequencing for *Pseudomonas* isolates and 16S and *gyrB* sequencing for *Xanthomonas* isolates; Partial sequences of approximately 1300bp for 16S and 820bp for *gyrB* sequencing.

[††] Sequences deposited in GenBank: WHRI 8848 16S sequence (KX518636) and *gyrB* sequence (KX523290); WHRI 8853 16S sequence (KX518637) and *gyrB* sequence (KX523291)

[§] *Pseudomonas cichorii* is an economically important plant pathogen with a wide host range (Ramkumar *et al.*, 2015), although this isolate from watercress failed to produce any disease in the plants tested. *Pseudomonas* species such as *P. fluorescens* and *koreensis* have been found in soils and water and are generally not associated with plant diseases (Kwon *et al.*, 2003, Paulsen *et al.*, 2005).

[§§] Isolates described in Vicente et al. (2001) and Vicente et al. (2006).

[#] Isolate described in Opio et al. (1996).

Table 2. Selected Biolog microplate GEN III carbohydrate tests that differentiate the watercress isolates from 21 *Xanthomonas* species*.

Test	Species																					
	X. sacchari	*X. pisi*	*X. melonis*	*X. campestris*	*X. codiaei*	*X. floridensis* (WHRI 8844, 8846A, 8848 and 8851)	*X. nasturtii* (WHRI 8853 and 8930A)	*X. axonopodis*	*X. arboricola*	*X. maliensis*	*X. cynarae*	*X. cassavae*	*X. vesicatoria*	*X. cucurbitae*	*X. fragariae*	*X. bromi*	*X. translucens*	*X. hyacinthy*	*X. hortorum*	*X. theicola*	*X. vasicola*	*X. oryzae*	*X. albilineans*
Propionic acid	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Saccharic acid	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Formic acid	+	+	-	-	-	(+)	-	-	+	v	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Ketobutyric acid	+	+	+	+	+	(+)	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
L-Aspartic acid	+	+	-	-	+	(+)	- / (+)	-	+	v	+	-	-	+	+	+	+	+	-	-	-	-	-
D-Raffinose	+	+	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-

* Summary data from Trébaol et al. (2000) based on results by Vauterin et al. (1995) for 20 *Xanthomonas* species and Triplett et al. (2015) for *X. maliensis*.
+, positive reaction; (+), weak reaction; -, negative reaction; v, variable

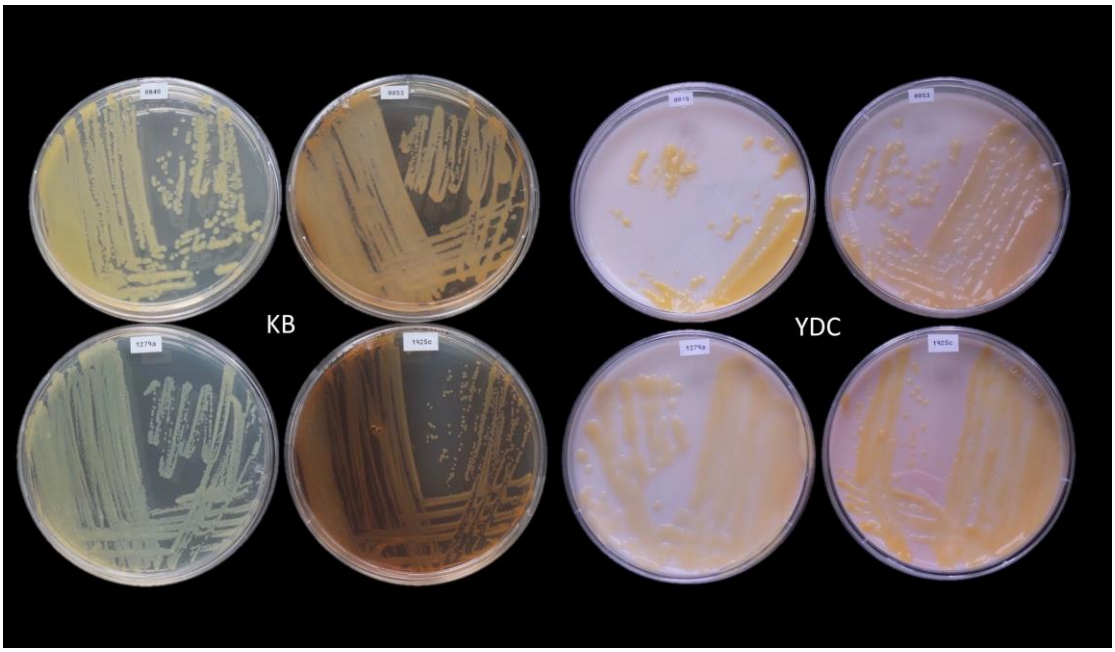

Fig. 1. *Xanthomonas floridensis* (WHRI 8848), *X. nasturtii* (WHRI 8853), *X. campestris* pv. *campestris* (WHRI 1279A) and *X. axonopodis* pv. *phaseoli* (WHRI 1925C) in plates with King's B agar (KB) and yeast dextrose calcium carbonate agar (YDC) media three days after sub-culturing.

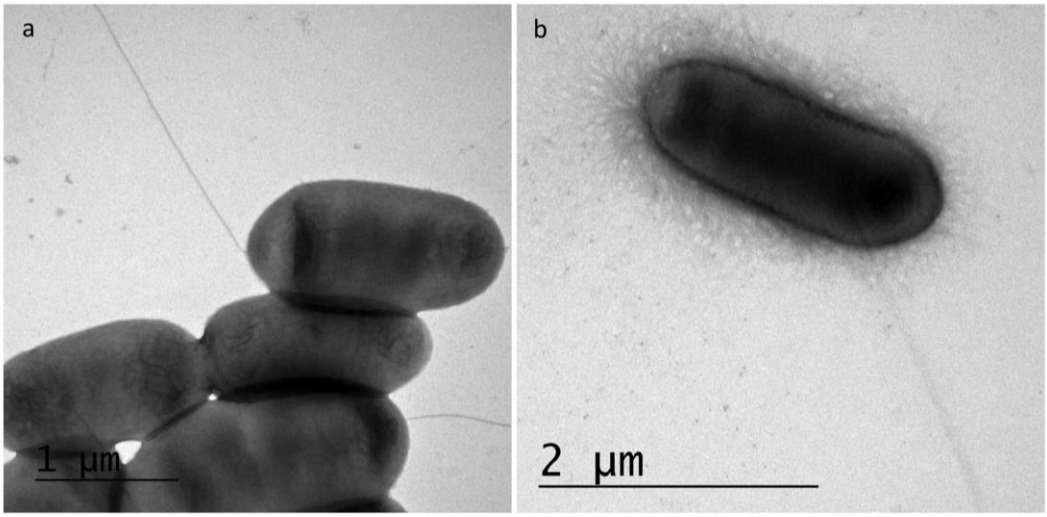

Fig. 2. Bacterial cells negatively stained and photographed under a JEM-2200FS Field Emission Electron Microscope (JEOL, Watchmead, Hertfordshire, UK) at the Life Sciences Imaging Suite, University of Warwick. Strains were grown in KB plates for 48h at 28°C and turbid suspensions were made in sterile water with 0.1% bacitracin; drops of 10µl were loaded onto formvar/carbon grids, blotted after one minute and stained with 2% uranyl acetate for 1 min.
(a) Strain WHRI 8848 (*Xanthomonas floridensis*) (b) Strain WHRI 8853 (*X. nasturtii*).

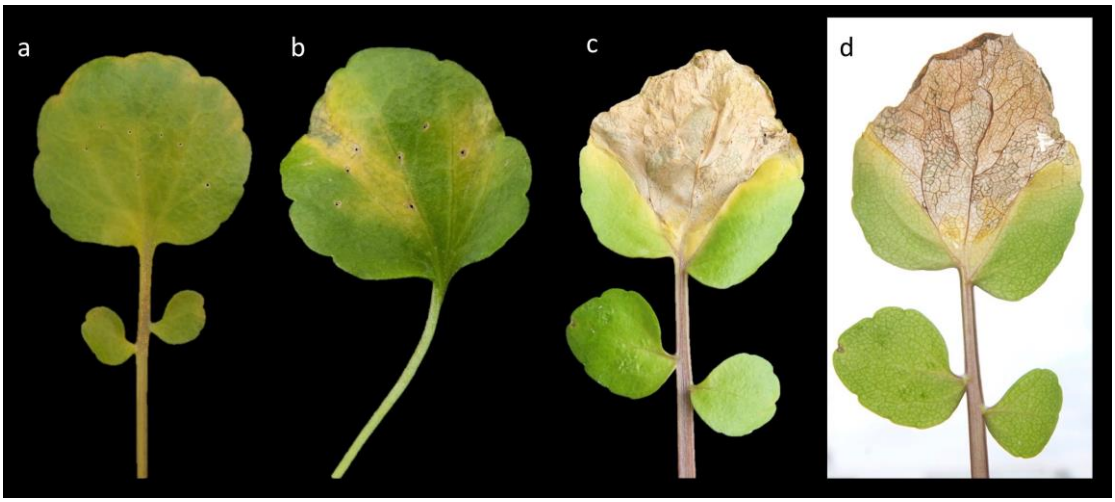

Fig. 3. Watercress inoculated with *Xanthomonas* spp. obtained from watercress. (a) Symptomless watercress (cv. Hampshire) following inoculation with *X. floridensis* (WHRI 8848) (b) disease symptoms in watercress (large leaved improved) inoculated with *X. nasturtii* WHRI 8853 (c) and (d) disease symptoms in watercress (cv. Hampshire) inoculated with the strain WHRI 8930A with black background and against the light respectively.

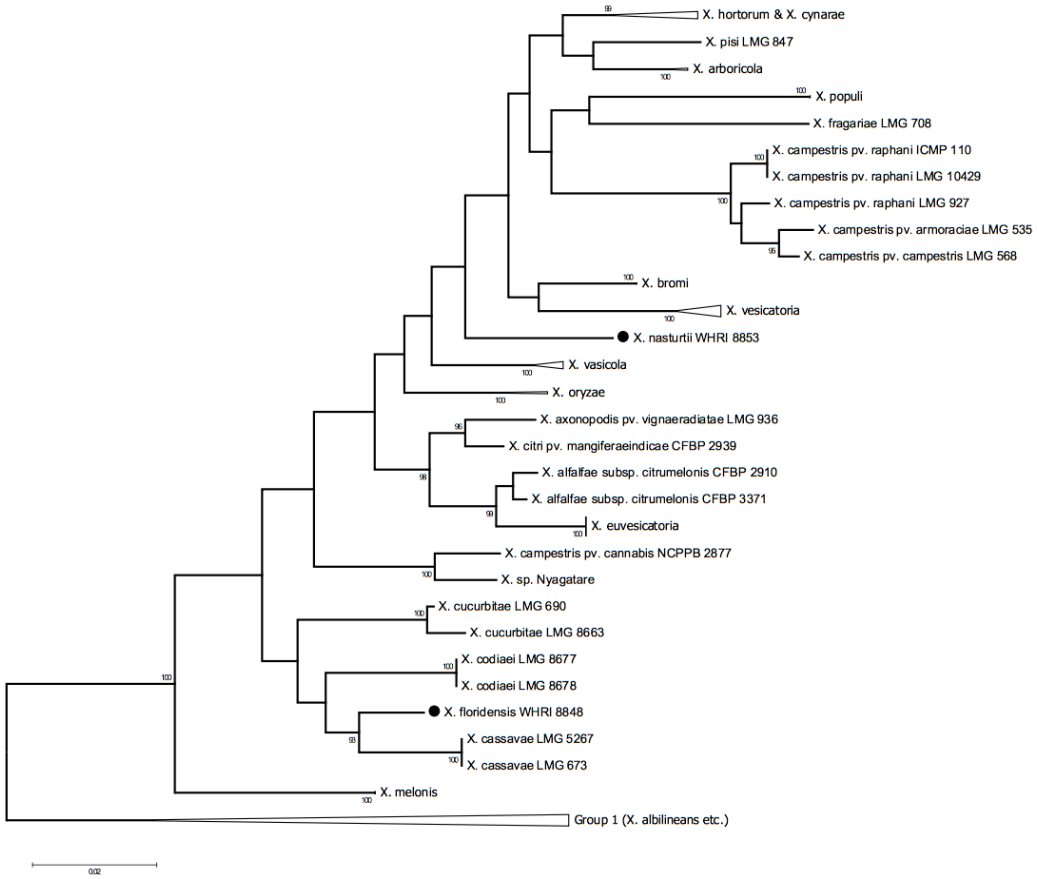

Fig. 4. Phylogenetic positions of strains of *Xanthomonas floridensis* (WHRI 8848) and *X. nasturtii* (WHRI 8853) based on multi-locus sequence analysis (MLSA). Nucleotide sequence data for four loci (*atpD, dnaK, efp* and *gyrB*) were obtained from a previously published MLSA study (Hamza *et al.*, 2012) and augmented with homologous sequences from the published genome assemblies of WHRI 8848 and 8853 as well as *X. campestris* pv. *cannabis* (Jacobs *et al.*, 2015) and strain Nyagatare (Aritua *et al.*, 2015), which have recently been proposed to belong to a new species *X. cannabis* (Jacobs *et al.*, 2015). Sequences were aligned using MAFFT and the resulting multi-sequence alignment was used to generate a Maximum-Likelihood tree using MEGA7 (Tamura *et al.*, 2013).