Short Communication

Xanthomonas citri pv. *citri* Findings in Citrus Fruits Imported in the Netherlands

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Abstract

Xanthomonas citri pv. *citri* and pv. *aurantifolii* are responsible for citrus bacterial canker. The trade of citrus fruit is recognized as one of the seven *X. citri* pv. *citri* introduction pathways. Under the current European Union phytosanitary measures, the likelihood of these *X. citri* pathovars being introduced into the Mediterranean region via infected fruit is rated as unlikely. To closely monitor the commercial trade flow of citrus fruit, imported lots that contained fruits with citrus bacterial canker symptoms were quarantined, and a sample was sent for laboratory analysis. Survey results revealed the presence of viable and highly pathogenic *X. citri* pv. *citri* isolates in 97 infected lots between 2013 and 2022, whereas *X. citri* pv. *aurantifolii* was never found. These *X. citri* pv. *citri*-infected lots primarily concerned *Citrus latifolia* (64), *C. hystrix* (17), and *C. grandis* (11) fruits, mainly from South America but also from several Asian countries. Additionally, a genetic characterization of these *X. citri* pv. *citri* isolates demonstrated that, although they originated from different countries, years, and *Citrus* species, they were all highly related, with average nucleotide identity scores ranging from 99.98 to 100%. These intercepted lots containing symptomatic citrus fruits due to the presence of *X. citri* pv. *citri* underlines the importance of continued vigilance by both the exporting and importing countries. Import inspections of the citrus fruit trade flow for the presence of citrus bacterial canker symptoms and subsequent laboratory testing remain significant tools for assessing the long-distance dispersal of *X. citri* pv. *citri*.

Keywords: citrus bacterial canker (CBC), imported citrus fruit lots, pathogen identification, survey, whole-genome sequencing (WGS), *Xanthomonas citri* pv. *citri*

Xanthomonas citri is a plant-pathogenic bacterial species of which two pathovars, pv. *citri* and pv. *aurantifolii*, are responsible for citrus bacterial canker (CBC). CBC presents a risk to the Mediterranean citrus-producing areas of the European Union (EU) due to favorable environmental conditions, the presence of numerous susceptible hosts (Dalla Pria et al. 2006), and the wide presence of the Asian citrus leafminer, *Phyllocnistis citrella* (EFSA et al. 2019). Population sizes of *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* in symptomatic citrus fruit can range from 10^5 to 10^7 viable cells per fruit lesion (Civerolo 1984; Gottwald et al. 2009), whereas in asymptomatic citrus fruit, it might not be detectable, as was demonstrated for asymptomatic Satsuma mandarin fruits (Zema et al. 2018). If infected citrus fruit arrives in the Mediterranean region during weather conditions that favor disease establishment and spread, such as rain and wind, as well as thunderstorms (EFSA et al. 2019), bacterial cells might successfully access the stomata or wounds of young vegetative tissues in citrus trees, which may lead to CBC high incidence and severity (Bock et al. 2010; Parker et al. 2008), especially during the susceptible growing period (early fruiting) (Juhasz et al. 2013). Due to the economic threat posed by these *X. citri* pathovars to citrus production and their absence in the Mediterranean region, they have a quarantine status in the EU (European Commission 2019). Therefore, EU legislation mandates targeted phytosanitary measures to reduce the risk of their introduction (European Commission 2019). Currently, the primary measures focus on prohibiting the import of *Citrus*, *Fortunella*, and *Poncirus* plants from third countries into the EU (European Commission 2019). Additionally, fruit import is only authorized from pest-free third countries or pest-free areas/production places in third countries. When fruits originate from third countries or areas/production places in third countries that are not free from these *X. citri* pathovars, integrated phytosanitary measures in a systems approach should be implemented. These range from cultural practices in the orchard to appropriate fruit treatments, both before and after harvest, with an approved chemical bactericidal that can reduce viable bacteria (Golmohammadi et al. 2007; Gottwald et al. 2009). The European Food Safety Authority (EFSA) indicates that these EU integrated phytosanitary measures are moderately to highly effective in reducing the risk of introduction of these *X. citri* pathovars, as evidenced by the fact that they have never been reported in the EU territory (EFSA 2014; EFSA et al. 2019; EPPO 2022). Specifically, the EFSA deems the likelihood of introducing these *X. citri* pathovars from infected citrus fruit to a suitable citrus plant unlikely (EFSA 2014).

CBC fruit symptoms mainly include brownish or grayish canker lesions of different size and structure (Fig. 1), often with a corky appearance, with elevated margins and an elevated or a sunken center (crater-like center) and sometimes surrounded by water-soaked margins and yellow halos. Other diseases of citrus fruits can cause

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symptoms that highly resemble the CBC symptoms, such as citrus scab (*Elsinoë fawcettii*, *E. australis*, or *E. citricola*) and Phaeoramularia fruit spot disease (*Pseudocercospora angolensis*) (EPPO 2023). Laboratory analysis is, therefore, required to confirm the presence of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* in these symptomatic citrus fruits. Although both *X. citri* pathovars cause similar lesions on the fruits, stems, and leaves of different *Citrus* species, the symptoms caused by *X. citri* pv. *citri* are much more devastating than those caused by *X. citri* pv. *aurantifolii* and can lead to death of the *Citrus* trees when infections are severe (EFSA et al. 2019). Additionally, the host range of *X. citri* pv. *aurantifolii* is rather restricted and primarily includes *Citrus* × *aurantifolia* and *Citrus* × *limon* and, to a lesser extent, *Citrus* × *aurantium*, *Citrus* × *limonia*, *C. maxima*, *C. medica*, and *Citrus* × *jambhiri*, whereas all *Citrus* spp. and several other rutaceous genera are considered main hosts of *X. citri* pv. *citri* (EPPO 2023). These two elements might explain why *X. citri* pv. *aurantifolii* has not been reported worldwide in the past decade (EPPO 2023; Fonseca et al. 2019). Phylogeographical analyses have demonstrated that *X. citri* pv. *citri* probably originated from India (Patané et al. 2019). From there, the pathogen expanded to other continents in the 20th century. The current worldwide distribution of *X. citri* pv. *citri* includes countries in Asia, Africa, North and South America, Oceania (EPPO 2022), and, more recently, the Middle East (Ibrahim et al. 2023). Many countries in these continents with pest-free status areas export citrus fruit to the EU. Nevertheless, reports indicate an ongoing expansion of the pathogen into new areas (Osdaghi 2022).

The import of citrus fruit in the Netherlands complies with the requirements of EU legislation and represents the highest import volume within the EU (European Commission 2019; 2022). The commercial trade of citrus fruit has been recognized by the EFSA as one of the seven *X. citri* pv. *citri* introduction pathways (EFSA 2014). This is supported not only by the large volume of citrus commodities imported into risk assessment areas in the EU but also by the successful survival of *X. citri* pv. *citri* in fruit canker lesions after pre- and postharvest fruit treatments and during fruit transport under cool conditions (Golmohammadi et al. 2007; Gottwald et al. 2009). To closely monitor this trade flow, imported lots of citrus fruit from outside the EU undergo visual inspection for the presence of CBC symptoms upon entering the Netherlands, as mandated by the EU (European Commission 2019; Mavrodieva et al. 2004). Lots that contain citrus fruits with symptoms that could indicate an *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* infection are quarantined, and a sample of symptomatic fruits is sent to the laboratory for official analysis. In this paper, we present the survey results for *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* on symptomatic citrus fruits imported into the Netherlands in the period of 2013 to 2022. Additionally, we provide the results of the genetic characterization of the pathogenic isolates associated with these symptomatic citrus fruits.

In the period of 2013 to 2022, an increase in the number of imported citrus fruit lots from outside the EU into the Netherlands was observed, from 29,700 lots in 2013 to more than 46,000 lots in 2022 (data retrieved from the Dutch national system CLIENT-import), with a peak of more than 50,000 lots in 2021 (Table 1; Fig. 1). This increase was most pronounced for fruits of *C. sinensis*, *C. reticulata*, *Citrus* × *limon*, and *Citrus* × *latifolia*, followed by *Citrus* × *clementina*. These citrus fruit lots originate mainly from South America but also include lots from several countries in Asia. The number of lots containing symptomatic citrus fruit, matching the expected CBC symptomatology and, therefore, included for the

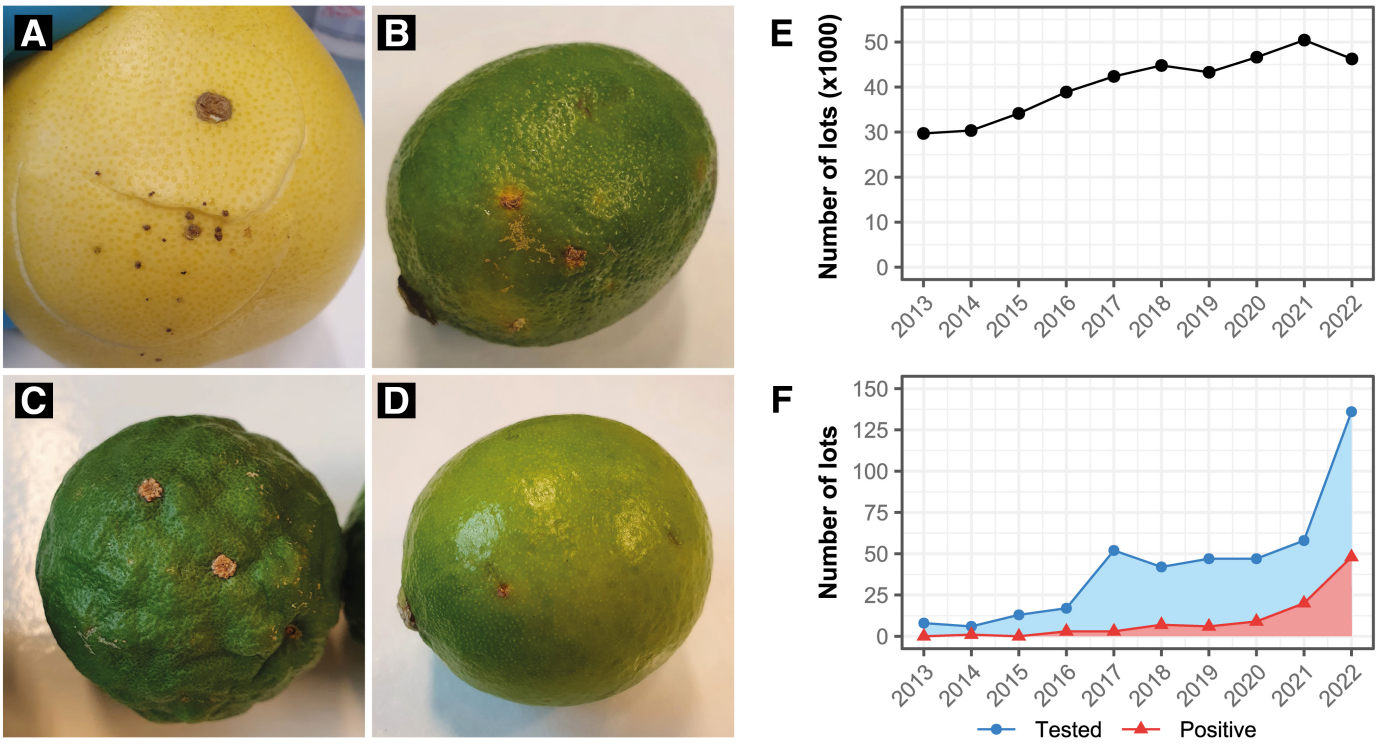

FIGURE 1

A to D, Typical symptoms (brown corky lesions) of *Xanthomonas citri* pv. *citri* infections on citrus fruit A, *Citrus grandis*; B, *Citrus latifolia*; C, *Citrus hystrix*; and D, *Citrus latifolia* upon import into the Netherlands. **E and F,** Overview of the number of citrus fruit lots and infected samples found in the Netherlands between the years 2013 and 2022: E, total number of citrus fruit lots imported into the Netherlands; F, number of symptomatic citrus fruit lots sent to the laboratory for analysis (blue circles) and number of symptomatic citrus fruit lots infected with *X. citri* pv. *citri* (red triangles).

laboratory analysis, increased from 8 in 2013 to 136 in 2022 (Fig. 1F). Small pieces of citrus fruit canker lesions, excised from whole fruits, were incubated in 0.01 M phosphate buffer (pH 7) at room temperature for 30 min. A molecular detection test targeting only *X. citri* pv. *citri* (Robène et al. 2020) was performed. When found to be negative, it was followed by another molecular detection test targeting both *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* (Mavrodieva et al. 2004). DNA isolation from fruit extracts was performed using the QuickPick Plant DNA Kit (Bio-Nobile) in conjunction with the Kingfisher Flex System (Thermo Fisher Scientific) for automation purposes. Because acquisition of a culture through isolation on nutrient media is an accurate way to assess living cells of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* from a canker fruit lesion, analysis of the symptomatic fruits included isolation on Wilbrink's and YPG media (EPPO 2023). A volume of 20 µl of the fruit extract was plated onto both media (EPPO 2023). The plates were incubated at 28°C for 2 to 4 days, and colonies exhibiting typical *X. citri* morphology were purified on Wilbrink's medium. The *X. citri* pv. *citri* colonies ranged from light yellow to bright yellow, varying in size and sliminess (EPPO 2023).

Obtaining a culture through isolation of the bacteria from citrus fruit canker lesions on a plate allows for the full identification of the isolate in addition to detecting the presence of viable cells in these lesions. The aforementioned identification tests included real-time PCR (Robène et al. 2020). Homogenized pure cultures were subjected to a heat treatment at 95°C for 15 min. Following cooling on ice, the samples were centrifuged at 5,000 × *g* for 3 min, and the resulting supernatant was used as the PCR template. Additionally, an immunofluorescence test was performed for identification based on two polyclonal antisera, Loewe Cat. No. 07302, Control No. 281117 for *X. citri* pv. *citri* and Loewe Cat. No. 07303, C. 29199/260219 for *X. citri* pv. *aurantifolii*, as described previously (EPPO 2023). These antisera presented high inclusivity only when used together; however, their exclusivity was rather poor. Therefore, in specific cases, sequence analysis of the *gyr*B and *avrBs2* loci (Constantin et al. 2016) was also performed on DNA isolated from axenic cultures, using either the High Pure PCR Template Preparation Kit (Roche) or the DNeasy Blood & Tissue Kit (QIAGEN).

Based on the different detection and identification tests performed, only infections with *X. citri* pv. *citri* were confirmed among the survey samples. No isolates of *X. citri* pv. *aurantifolii* were detected, which aligns well with the apparent absence of this pathovar in recent years (EFSA et al. 2019). Survey results further demonstrated that the percentage of *X. citri* pv. *citri*-infected citrus fruit lots, as compared with the total number of citrus fruit lots in the period of 2013 to 2022, rose from 0% in 2013 and 0.003% in 2014 to 0.1% in 2022, corresponding to 97 lots between 2013 and 2022 (Table 1). In 2022, 48 citrus fruit lots matching the expected CBC symptomatology were infected with *X. citri* pv. *citri*. These *X. citri* pv. *citri*-infected lots in 2013 to 2022 primarily referred to fruits of *C. latifolia* (64), *C. hystrix* (17), and *C. grandis* (11) (Table 1). An increase in *X. citri* pv. *citri*-infected *C. latifolia* (Tahiti lime) lots was observed in 2021 (16) and 2022 (43), concerning *C. latifolia* lots from specific South American countries. This increase could potentially be attributed to adopted legislation in these countries. For example, a new CBC control legislation implemented in 2018 in Brazil (Behlau 2021) allows for the retention of citrus canker-infected trees in the field in areas with an intermediate or high disease incidence. The effect of the retention of citrus canker-infected trees on the epidemiology of *X. citri* pv. *citri* in these and neighboring areas has not been studied. Additionally, the relatively high number of *X. citri* pv. *citri*-infected *C. hystrix* (kaffir lime) samples from countries in Asia is remarkable because of the relatively low number of *C. hystrix* imported fruit lots (Table 1) on an annual basis (e.g., number of fruit lots in 2020: *C. hystrix* 55; *C. grandis* 3,343; *C. latifolia* 5,295).

To assess the virulence of the *X. citri* pv. *citri* isolates obtained in this survey, a detached leaf pathogenicity assay was conducted. Two young leaves of *Citrus* × *microcarpa* and two young leaves of either *C.* × *limonia* or *C. sinensis* were infiltrated with a thick suspension of bacterial cells in 0.01 M phosphate buffer (pH 7), as previously described (EPPO 2023). The leaves were then incubated at a constant temperature of 22°C and 80% humidity, with a 12-h light/dark cycle for 2 weeks. Following symptom evaluation, the pathogen was re-isolated on Wilbrink's medium followed by real-time PCR (Robène et al. 2020) as described above to confirm its identity. The results of the detached leaf assay demonstrated that all 97 *X. citri* pv. *citri* isolates obtained in this survey in the period of 2013 to 2022 were highly virulent, as the infiltrated young leaves of *Citrus* × *microcarpa* and *C.* × *limonia* or *C. sinensis* exhibited high disease incidence (>90%) 2 weeks postinfiltration.

Additionally, the genetic characterization of 31 representative *X. citri* pv. *citri* isolates (Table 2) found in the symptomatic citrus fruits imported into the Netherlands in 2013 to 2022, and

TABLE 1
Total number of lots per citrus species per year imported into the Netherlands and number of symptomatic citrus fruit lots infected with *Xanthomonas citri* pv. *citri* per citrus species per year (in parentheses) between 2013 and 2022

Year	*Citrus sinensis*	*Citrus reticulata*, all variants	*Citrus* × *limon*	*Citrus* × *latifolia*	*Citrus* × *paradisi*	*Citrus grandis* and *maxima*	*Citrus hystrix*	Other species	Total all species
2013	11,192	3,743	3,071	3,625	4,062	3,301	57	649	29,700
2014	12,145 (1)	4,072	2,584	3,891	3,629	3,160	59	809	30,349 (1)
2015	13,675	4,692	3,189	4,416	3,875	3,508	55	736	34,146
2016	14,868	5,360	4,766	4,601	4,256	3,739 (1)	58 (2)	1,217	38,865 (3)
2017	17,522	5,437	3,959 (1)	4,844	4,656	4,610 (1)	69 (1)	1,266	42,363 (3)
2018	17,524	6,166	4,584	5,661	5,011	4,544 (2)	110 (4)	1,191 (1)	44,791 (7)
2019	17,033	5,723	4,290	5,308 (1)	4,871	4,853 (2)	64 (2)	1,140 (1)	43,282 (6)
2020	17,574	6,883	6,060	5,295 (4)	4,667	5,067 (1)	55 (4)	1,027	46,628 (9)
2021	20,329	8,460	7,136	5,767 (16)	4,560 (1)	2,993 (1)	52 (2)	1,130	50,427 (20)
2022	17,418	7,248	7,113	6,291 (43)	3,987	3,225 (3)	49 (2)	916	46,247 (48)
Total infected	1	0	1	64	1	11	17	2	97

TABLE 2
Metadata of isolates for which assemblies were generated in this study[a]

Collection number	Species	Origin	Host plant	Year	Assembly accession number	Reference
PD 3791	*Xanthomonas citri* pv. *citri*	Unknown	*Citrus hystrix*	2000	GCA_963669695.1	This study
PD 3793	*X. citri* pv. *citri*	Unknown	*Citrus hystrix*	2000	GCA_963669625.1	This study
LMG 683	*X. citri* pv. *citri*	New Zealand	*Citrus sinensis*	1956	GCA_963669845.1	–
LMG 9176, XC59	*X. citri* pv. *citri*	Brazil	*Citrus aurantifolia*	–	GCA_963669535.1	Vauterin et al. 1991
LMG 9179, XC64	*X. citri* pv. *aurantifolii*	Argentina	*Citrus limon*	1982	GCA_963669675.1	Vauterin et al. 1991
LMG 9182, XC90	*X. citri* pv. *aurantifolii*	Mexico	*Citrus aurantifolia*	–	GCA_963669685.1	Vernière et al. 1998
NCPPB 409	*X. citri* pv. *citri*	New Zealand	*Citrus limon*	1956	GCA_963669875.1	–
PD 3895	*X. citri* pv. *citri*	Unknown	*Citrus aurantifolia*	2000	GCA_963669895.1	This study
PD 3896	*X. citri* pv. *citri*	Unknown	*Citrus hystrix*	2000	GCA_963669885.1	This study
PD 4106	*X. citri* pv. *citri*	Argentina	*Citrus reticulata*	2001	GCA_963669725.1	This study
PD 4399	*X. citri* pv. *citri*	Argentina	*Citrus sinensis*	2002	GCA_963669915.1	This study
PD 4774	*X. citri* pv. *citri*	Thailand	*Citrus* sp.	2004	GCA_963669745.1	This study
PD 5427	*X. citri* pv. *citri*	Thailand	*Citrus* sp.	2007	GCA_963669795.1	This study
PD 5812	*X. citri* pv. *citri*	Unknown	*Citrus* sp.	2009	GCA_963669735.1	This study
PD 5839	*X. citri* pv. *citri*	Thailand	*Citrus* sp.	2010	GCA_963669785.1	This study
PD 6311, XC 62	*X. citri* pv. *citri*	Japan	*Citrus* sp.	1978	GCA_963669835.1	Vernière et al. 1998
PD 6312, XC 69	*X. citri* pv. *aurantifolii*	Argentina	*Citrus* sp.	1979	GCA_963669495.1	Vernière et al. 1998
PD 6313, XC 205	*X. citri* pv. *citri*	Southwest Asia	*Citrus aurantifolia*	–	GCA_963669765.1	Sun et al. 2004
PD 6314, XC 341	*X. citri* pv. *aurantifolii*	Brazil	–	1982	GCA_963669755.1	Leite et al. 1994
LMG 9322	*X. citri* pv. *citri*	United States	*Citrus aurantifolia*	1989	GCA_963669775.1	Vernière et al. 1998
PD 6982	*X. citri* pv. *citri*	Uruguay	*Citrus sinensis*	2014	GCA_963669805.1	This study
PD 7215	*X. citri* pv. *citri*	Indonesia	*Citrus hystrix*	2016	GCA_963669705.1	This study
PD 7280	*X. citri* pv. *citri*	China	*Citrus maxima*	2016	GCA_963669525.1	This study
PD 7301	*X. citri* pv. *citri*	Indonesia	*Citrus hystrix*	2017	GCA_963669565.1	This study
PD 7340	*X. citri* pv. *citri*	Uruguay	*Citrus limon*	2017	GCA_963669665.1	This study
PD 7365	*X. citri* pv. *citri*	China	*Citrus maxima*	2017	GCA_963669965.1	This study
PD 7366	*X. citri* pv. *citri*	China	*Citrus maxima*	2018	GCA_963669715.1	This study
PD 7391	*X. citri* pv. *citri*	Unknown	*Citrus hystrix*	2018	GCA_963669515.1	This study
PD 7392	*X. citri* pv. *citri*	Indonesia	*Citrus hystrix*	2018	GCA_963669935.1	This study
PD 7435	*X. citri* pv. *citri*	Indonesia	*Citrus amblycarpa*	2018	GCA_963669555.1	This study
PD 7450	*X. citri* pv. *citri*	China	*Citrus grandis*	2018	GCA_963669545.1	This study
Kr 4819	*X. citri* pv. *citri*	Indonesia	*Citrus hystrix*	2019	GCA_963669595.1	This study
PD 7479	*X. citri* pv. *citri*	Indonesia	*Citrus limonia*	2019	GCA_963669585.1	This study
LMG 9160, F1	*X. euvesicatoria* pv. *citrumelonis*	United States	*Poncirus trifoliata* × *Citrus sinensis*	1984	GCA_963669605.1	Jalan et al. 2011; Vauterin et al. 1991
LMG 9161, F3	*X. euvesicatoria* pv. *citrumelonis*	United States	*Citrus paradisi*	–	GCA_963669645.1	Vauterin et al. 1991
LMG 9325	*X. euvesicatoria* pv. *citrumelonis*	United States	*Citrus* sp.	–	GCA_963669575.1	–
NCPPB 1759	*X. citri*	India	*Feronia elephantacum*	–	GCA_963669615.1	–
NCPPB 3213	*X. citri*	India	*Aegle marmelos*	1980	GCA_963669635.1	Jalan et al. 2011; Vauterin et al. 1991
CFBP 3842, F56	*X. euvesicatoria*	United States	*Poncirus trifoliata* × *Citrus paradisi*	–	GCA_963669655.1	Vauterin et al. 1991
CFBP 3843, F231	*X. euvesicatoria*	United States	*Citrus paradisi*	–	GCA_963669855.1	Vauterin et al. 1991
CFBP 3844, F294	*X. euvesicatoria*	United States	*Citrus paradisi*	–	GCA_963669865.1	Vauterin et al. 1991
PD 7641	*X. citri* pv. *citri*	Vietnam	*Citrus latifolia*	2020	GCA_963669905.1	This study
PD 8002	*X. citri* pv. *citri*	Brazil	*Citrus latifolia*	2021	GCA_963669945.1	This study
PD 8277	*X. citri* pv. *citri*	Brazil	*Citrus latifolia*	2022	GCA_963669825.1	This study
PD 8327	*X. citri* pv. *citri*	Brazil	*Citrus latifolia*	2022	GCA_963669925.1	This study
PD 8332	*X. citri* pv. *citri*	Vietnam	*Citrus hystrix*	2022	GCA_963669505.1	This study
PD 8348	*X. citri* pv. *citri*	Brazil	*Citrus latifolia*	2022	GCA_963669485.1	This study
PD 8351	*X. citri* pv. *citri*	Vietnam	*Citrus latifolia*	2022	GCA_963669815.1	This study
PD 8358	*X. citri* pv. *citri*	China	*Citrus maxima*	2022	GCA_963669955.1	This study

[a] Isolates of *Xanthomonas citri* pv. *citri* identified in the symptomatic citrus fruit lots upon import into the Netherlands are highlighted in bold.

incidentally in the years 2000 to 2010, was performed by generating their near-complete genomes using Illumina sequencing. These isolates were obtained from symptomatic fruit originating from different countries, years, and host plants of citrus. In addition to these *X. citri* pv. *citri* isolates, two *X. citri* isolates from India (of unknown pathovar) and six *X. euvesicatoria* isolates (including three of pv. *citrumelonis*) isolated from *Poncirus trifoliata* × *C. sinensis* and *C. paradisi* were also included in the sequencing analysis (Table 2). The DNA extracts were sequenced by GenomeScan using NovaSeq 6000 as previously described (Van Valkenburg et al. 2022). The obtained Illumina reads are deposited under BioProject accession number PRJEB65884. Next, the reads were assembled and an-

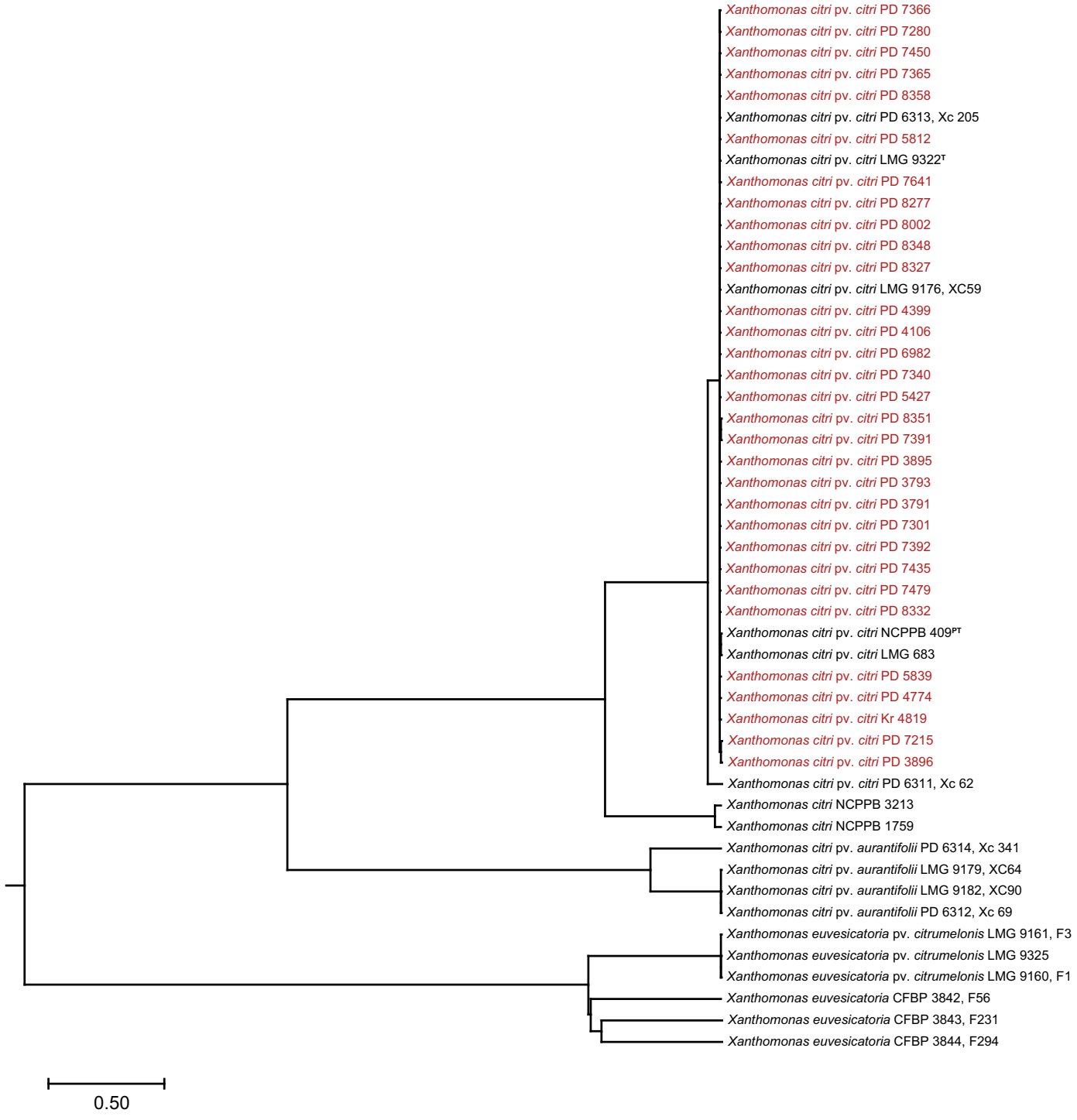

FIGURE 2

Phylogenetic tree depicting the genetic relationships among *Xanthomonas* isolates based on average nucleotide identity values. Isolates of *X. citri* pv. *citri*, identified in symptomatic citrus fruit lots upon import into the Netherlands, are highlighted in red. The scale bar illustrates the percentage difference in average nucleotide identity values. T = type strain; PT = pathotype strain of *X. citri* pv. *citri*.

notated using the Read Assembly and Annotation Pipeline Tool (RAPT) available at https://www.ncbi.nlm.nih.gov/rapt. For assembly, RAPT uses strategic k-mer extension for scrupulous assemblies (SKESA, version 2.5.0), whereas the annotation step utilizes the NCBI prokaryotic genome annotation pipeline (PGAP, version 6.5) (Souvorov et al. 2018; Tatusova et al. 2016). To perform whole-genome alignments, the annotated genomes were processed using the Whole Genome Alignment plugin within CLC Genomics Workbench 23.0.2 (QIAGEN). Subsequently, average nucleotide identity comparisons were generated using the same plugin. Based on these average nucleotide identity scores, a tree was constructed using the unweighted pair group method with arithmetic mean method. The tree (Fig. 2) shows that, although the 31 *X. citri* pv. *citri* isolates originated from different countries, years, and citrus host plants (Table 2), they were all highly related, with average nucleotide identity scores ranging from 99.98 to 100%.

Large volumes of citrus fruits are traded worldwide, and, when infected with *X. citri* pv. *citri*, they represent a source of inoculum for introducing the pathogen into new areas (Ali et al. 2023; Osdaghi 2022). Nevertheless, pre- and postharvest disinfected citrus fruits are not considered to be a significant pathway for *X. citri* pv. *citri* to reach and infect susceptible citrus and become established in canker-free areas (Gottwald et al. 2009). The increasing number of intercepted lots containing symptomatic citrus fruits with canker lesions found in the Netherlands, however, underlines the importance of continued vigilance by both the exporting and the importing countries. The successful isolation of living cells of *X. citri* pv. *citri* from the symptomatic citrus fruit samples received for laboratory analysis shows the presence of viable bacteria. This, together with the high virulence of the *X. citri* pv. *citri* isolates, as demonstrated in the pathogenicity assays, justifies the importance of continued vigilance. As was recently demonstrated in Swingle citrumelo, selected bactericidal treatments targeting *X. citri* pv. *citri*, especially under the concentrations currently approved for citrus fruit disinfection, are not completely effective (Redondo et al. 2015) due to the biofilm formation that protects the bacterial cells from exposure to these bactericides. Consequently, bactericide treatments should not only focus on the elimination of bare *X. citri* pv. *citri* cells but also include the potential to disrupt biofilms (Redondo et al. 2015). Recent work demonstrated a linear relationship between citrus fruit lesion size caused by *X. citri* pv. *citri* and bacterial survival within lesions, both before and after harvest, with larger lesions posing a greater risk (Luo et al. 2020). More research is needed to fully address the efficacy of the current bactericidal treatments used for citrus fruit disinfection in the presence of biofilms, at both the pre- and postharvest stages.

The citrus processing industry produces large quantities of citrus peel waste (Kim et al. 2022; Maqbool et al. 2023; Russo et al. 2021; Sharma et al. 2022; Zema et al. 2018) that, despite its valuable compounds (pectin, flavonoids, fibers, sugar, etc.), is often not recycled but ends up in landfills (Russo et al. 2021; Zema et al. 2018). Within the Mediterranean region, citrus orchards neighboring areas where potentially infected fruit or citrus peel waste from potentially infected fruit is being discarded are considered of higher relative risk than citrus orchards that do not neighbor such areas (EFSA et al. 2019). However, *X. citri* pv. *citri* seemed unable to survive when in contact with the disrupted oil glands of the fruit during the production of the citrus peel waste (Fulton and Bowman 1929). Placing cull piles of infected grapefruits or lemons or placing infected fruits near healthy grapefruit plants did not result in infection of these healthy grapefruit plants under normal natural conditions (Gottwald et al. 2009). A low risk of spread was identified when severely infected cull piles were used under simulation of extreme weather (wind and rain) conditions (Gottwald et al. 2009). In this situation, a single CBC lesion developed on a grapefruit plant positioned in the wind direction from the cull pile. Similar results were found when infected mandarins were placed in navel orange orchards (Shiotani et al. 2009). However, execution of such simulation experiments is rather difficult, and these experiments were performed under conditions that might not always be representative of all citrus orchard environments. Extreme weather conditions, including thunderstorms, regularly occurred in the Mediterranean region in the last few years and are expected to occur more in the future as a result of climate change. This phenomenon can facilitate successful access of bacterial cells into stomata or wounds, caused during these thunderstorms on citrus trees, which can lead to *X. citri* pv. *citri* infection (Bock et al. 2010; Parker et al. 2008). More experimental data to fully address the role of infected citrus fruit and peel as inoculum sources when discarded in the vicinity of citrus trees need to be generated, especially under extreme climate change scenarios.

In summary, import inspections of the citrus fruit trade flow for the presence of CBC symptoms and subsequent laboratory testing remain significant tools in reducing the potential risk of the long-distance dispersal of *X. citri* pv. *citri*, as evidenced by the virulent *X. citri* pv. *citri* populations present in the symptomatic citrus fruit lots imported. Imported citrus fruit lots found to be infected with *X. citri* pv. *citri* are either shipped back to the exporting country or are discarded according to the phytosanitary regulations.

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