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# Aggressive Emerging Pathovars of *Xanthomonas arboricola* Represent Widespread Epidemic Clones Distinct from Poorly Pathogenic Strains, as Revealed by Multilocus Sequence Typing

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**Deep and comprehensive knowledge of the genetic structure of pathogenic species is the cornerstone on which the design of precise molecular diagnostic tools is built. *Xanthomonas arboricola* is divided into pathovars, some of which are classified as quarantine organisms in many countries and are responsible for diseases on nut and stone fruit trees that have emerged worldwide. Recent taxonomic studies of the genus *Xanthomonas* showed that strains isolated from other hosts should be classified in *X. arboricola*, extending the host range of the species. To investigate the genetic structure of *X. arboricola* and the genetic relationships between highly pathogenic strains and strains apparently not relevant to plant health, we conducted multilocus sequence analyses on a collection of strains representative of the known diversity of the species. Most of the pathovars were clustered in separate monophyletic groups. The pathovars pruni, corylina, and juglandis, responsible for pandemics in specific hosts, were highly phylogenetically related and clustered in three distinct clonal complexes. In contrast, strains with no or uncertain pathogenicity were represented by numerous unrelated singletons scattered in the phylogenic tree. Depending on the pathovar, intra- and interspecies recombination played contrasting roles in generating nucleotide polymorphism. This work provides a population genetics framework for molecular epidemiological surveys of emerging plant pathogens within *X. arboricola*. Based on our results, we propose to reclassify three former pathovars of *Xanthomonas campestris* as *X. arboricola* pv. arracaciae comb. nov., *X. arboricola* pv. guizotiae comb. nov., and *X. arboricola* pv. zantedeschiae comb. nov. An emended description of *X. arboricola* Vauterin et al. 1995 is provided.**

As international trade and travel increase, emerging infectious diseases regularly threaten human health, as well as agricultural production (livestock and crops), and may be responsible for huge social, economic, and environmental damages. Our capability to quickly detect and identify the microorganisms responsible for these emerging diseases is critical to implement effective crop protection, sanitary measures, and regulations. The development of efficient and precise diagnostic tools relies on a stable and comprehensive classification. Providing a classification frame with names predictive of ecological, phenotypic, genotypic, and phylogenetic properties is the primary aim of taxonomic studies.

Sequencing of sets of housekeeping genes (multilocus sequence analysis [MLSA]) has become the standard today for phylogenetic analyses of bacterial species and was proposed as an alternative to DNA-DNA hybridizations for species delineation (1, 2). MLSA allows strain assignment at the species level (3–5) and gives rise to specialized databases (5–7). MLSA treats sequences at the nucleotide level for phylogenetic analysis, but the recorded data can also be converted into alleles, an approach known as multilocus sequence typing (MLST). MLST was introduced by Maiden et al. (8) to type bacterial pathogens and to identify the pathogens that are the origins of epidemics. Since then, it has been applied to many bacterial species and has been used in molecular epidemiology and microevolution analyses (5). MLST represents an easy, reproducible, and portable approach for pathogen identification at the infraspecies level and is also used to infer population genetic insights (9).

*Xanthomonas arboricola* is a bacterial species associated with plants that includes strains responsible for major diseases of stone and nut fruit trees. Plant-pathogenic strains are classified into pathovars, which are groups of strains responsible for the same disease in the same host range (10). The three most economically important pathovars within the species are pathovars pruni, corylina, and juglandis, responsible for bacterial spot of stone fruit trees, bacterial blight of hazelnut, and walnut blight, respectively. These three diseases were first reported in the early 20th century in the United States and are characterized by angular necrotic leaf spots and cankers on their respective hosts (11–13). Disease emergences were recently reported in several countries worldwide, such as Spain (14, 15), Italy, Serbia, Poland, Chile (16), Taiwan (17), and the United States (18). Consequently, these pathovars are recommended

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for regulation as quarantine pests in many countries (http://www.eppo.int/QUARANTINE/quarantine.htm; https://www.ippc.int/).

At the time of its description (19), *X. arboricola* encompassed five pathovars (pruni, corylina, juglandis, celebensis, and populi) plus strains previously classified as *Xanthomonas campestris* pv. poinsettiicola type C. In 2001, Janse et al. (20) described a new pathovar within the species, *X. arboricola* pv. fragariae, causing leaf blight of strawberry. Based on pathogenicity tests, virulence gene content, and genetic heterogeneity, the pathovar status of *X. arboricola* strains from strawberry was recently questioned (21). A phylogenetic analysis of the genus *Xanthomonas*, based on partial sequencing of the housekeeping gene *gyrB*, showed that the pathotype strains of *X. campestris* pv. arracaciae (22), *X. campestris* pv. guizotiae (23), and *X. campestris* pv. zantedeschiae (24) cluster near the type strain of *X. arboricola*, indicating that these three pathovars might be reclassified into *X. arboricola* (25). The same study showed that unclassified strains isolated from *Chrysanthemum* and clove belong to the *X. arboricola* clade. Xanthomonads isolated in New Zealand from diverse hosts, including *Magnolia*, also cluster with *X. arboricola* strains (26). These recent studies reveal the extended host range of *X. arboricola* and raise interesting questions about the phylogenetic relationships between these diverse strains isolated from a set of various hosts and strains of the historical pathovars (pruni, corylina, juglandis, and populi). Is classification within *X. arboricola*, inferred by *gyrB* sequencing, also supported by a multilocus approach? Do strains isolated from the same host cluster together, as expected under evolution driven by host adaptation?

MLSA-MLST approaches have been successfully used to describe the genetic structure of several *Xanthomonas* species, such as *X. campestris* (27), *Xanthomonas axonopodis* (28), and *Xanthomonas oryzae* (29). These studies reveal the importance of host specificity and pathogenicity traits in the population structure of these *Xanthomonas* species. It has also been shown that pathovar classification is strongly related to virulence-associated gene repertoires (29–31). Within *X. arboricola*, it was shown that the three major pathovars shared similar, yet slightly different, repertoires of type III effectors (T3Es) (32), which are the main virulence factors of xanthomonads (33). This result suggests a common origin for these three pathogens that needs to be investigated by phylogenetic approaches.

Here, we addressed the questions of the genetic structure of *X. arboricola* and the phylogenetic relationships of strains belonging to different pathovars that have so far been defined in the species using phylogenetic and molecular epidemiological methods. Based on analyses of partial sequences of seven housekeeping genes (MLST-MLSA), we showed that strains belonging to well-defined pathovars generally clustered in well-supported monophyletic groups. Strains of the three major pathovars, which are highly adapted to their respective host plants and have been responsible for pandemics over many decades, causing important economic losses in different production areas worldwide, clustered into three clonal complexes (CCs). Phylogenetic analyses supported the hypothesis of a shared common ancestor for the three pathovars. In contrast, strains isolated from various hosts that are weakly pathogenic or not pathogenic were genetically heterogeneous, and no genetic structure could be determined. Reclassification of *X. campestris* pv. arracaciae, *X. campestris* pv. guizotiae, and *X. campestris* pv. zantedeschiae in *X. arboricola* as *X. arboricola* pv. arracaciae comb. nov., *X. arboricola* pv. guizotiae comb. nov., and *X. arboricola* pv. zantedeschiae comb. nov. is proposed.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** A core collection of 97 strains of *Xanthomonas* representing the pathovars pruni, corylina, juglandis, fragariae, celebensis, populi, arracaciae, guizotiae, and zantedeschiae and *X. campestris* pv. poinsetticola type C, plus strains from diverse hosts not classified under pathovars (Table 1), was characterized by MLSA-MLST using partial nucleotidic sequences of seven housekeeping genes. They were chosen in order to maximize host and geographical origins and years of isolation. A few strains of the pathovars celebensis, guizotiae, zantedeschiae, and arracaciae are available in public microbial resource centers; all of them were used in this study. In addition, 160 strains deposited at the CIRM-CFBP (International Center for Microbial Resources—French Collection for Plant-Associated Bacteria, Angers, France) as *X. arboricola* were analyzed using partial nucleotide sequences of *gyrB* and *rpoD* (see Table S1 in the supplemental material). All the strains are preserved freeze-dried at the CIRM-CFBP. Bacterial strains were routinely cultured on YPGA medium (yeast extract, 7 g liter$^{-1}$; peptone, 7 g liter$^{-1}$; glucose, 7 g liter$^{-1}$; agar, 15 g liter$^{-1}$) for 2 to 4 days at 28°C.

**PCR and sequencing of protein-coding genes.** Amplification of partial sequences of seven protein-coding genes (*atpD* [ATP synthase β chain], *dnaK* [70-kDa heat shock protein], *efp* [elongation factor P], *fyuA* [transmembrane protein; Ton-B-dependent transporter], *glnA* [glutamine synthetase I], *gyrB* [DNA gyrase β subunit], and *rpoD* [RNA polymerase sigma 70 factor]) was performed as previously described (27, 28) using corresponding primers (Table 2). Both strands of PCR products were sequenced by Biogenouest (Nantes, France) and Genoscreen (Lille, France).

**Sequence acquisition and alignment.** Both strands of nucleotide sequences were edited, assembled, translated, aligned, and trimmed using Geneious software (Biomatters, Auckland, New Zealand). Amino acid alignments were used in order to produce codon-based nucleotide sequence alignments. The consensus sequences used to trim the sequences are shown in Table 2. The sequences were concatenated following the alphabetical order of the genes, ending in a sequence of 4,620 bp (bp 1 to 750 for *atpD*, 751 to 1509 for *dnaK*, 1510 to 1848 for *efp*, 1849 to 2601 for *fyuA*, 2602 to 3276 for *glnA*, 3277 to 4011 for *gyrB*, and 4012 to 4620 for *rpoD*).

**Sequence data analysis.** All summary statistics were calculated on the global data set. The number of polymorphic sites (*S*) and the number of haplotypes (Hap), as well as haplotype diversity (Hd) (34), nucleotide diversities ($\theta\pi$ and $\theta w$) (34, 35), neutrality indices of Tajima's *D* (36), Fu and Li's $D^*$, Fu and Li's $F^*$ (37), the number of nonsynonymous substitutions per nonsynonymous site (*dN*), and the number of synonymous substitutions per synonymous site (*dS*) (38), were estimated using DnaSP (39).

**Phylogenetic analyses.** Phylogenetic analyses were performed on individual gene sequences and on the concatenated data set. Strain CFBP 5241 of *X. campestris* pv. campestris was used to root trees. Neighbor-joining (NJ) trees were generated with MEGA version 5 (40) using the Kimura two-parameter model (41) and 1,000 bootstrap replicates. The model of evolution for maximum-likelihood (ML) analysis was determined using Modeltest 3.7 coupled with PAUP (42). Both the hierarchical likelihood ratio test (hLRT) and the standard Akaike information criterion (AIC) were determined to select a model. If given models were different, the phylogeny was inferred using the AIC results. ML trees were obtained with PhyML phylogeny software (43) using the best-fit nucleotide substitution model selected as described above and 1,000 bootstrap replicates. Trees were edited with MEGA 5. The Shimodaira-Hasegawa (SH) test (44) implemented in the DNAML program from PHYLIP (45) was used to test whether tree topologies based on each individual locus or on the concatenated sequences fell within the same confidence limits.

**TABLE 1** Bacterial strains used in this study

| Taxonomic name[a] | CFBP no.[d] | Other collection no.[f] | Host of isolation | Geographic origin | Yr of isolation | ST | CC |
|---|---|---|---|---|---|---|---|
| *X. arboricola* pv. pruni | CFBP 411 | ATCC 10016 | *Prunus persica* | United States | 1963 | 37 | 3 |
| | CFBP 2535$^{PT}$ | NCPPB 416, ICMP 51 | *Prunus salicina* | New Zealand | 1953 | 15 | 3 |
| | CFBP 3893 | | *Prunus persica* | Italy | 1989 | 37 | 3 |
| | CFBP 3898 | | *Prunus domestica* | United States | 1989 | 37 | 3 |
| | CFBP 3900 | | *Prunus persica* | United States | 1987 | 37 | 3 |
| | CFBP 3901 | | *Prunus armeniaca* | United States | 1987 | 37 | 3 |
| | CFBP 3921 | | *Prunus persica* | Italy | 1996 | 37 | 3 |
| | CFBP 5229 | | *Prunus* sp. | Argentina | 1996 | 15 | 3 |
| | CFBP 5529 | NCPPB 1607 | *Prunus persica* | Australia | 1964 | 37 | 3 |
| | CFBP 5580 | | *Prunus japonica* | France | 2000 | 37 | 3 |
| | CFBP 5722 | | *Prunus persica* | Brazil | 1991 | 37 | 3 |
| | CFBP 5723 | | *Prunus* sp. | Uruguay | NA[g] | 15 | 3 |
| | CFBP 5724 | | *Prunus amygdalus* | United States | NA | 15 | 3 |
| | CFBP 6653 | | *Prunus persica* | France | 2000 | 41 | 3 |
| | CFBP 7098 | | *Prunus domestica* | Spain | 2002 | 37 | 3 |
| | CFBP 7099 | | *Prunus domestica* | Spain | 2003 | 37 | 3 |
| | CFBP 7100 | | *Prunus dulcis* | Spain | 2006 | 37 | 3 |
| *X. arboricola* pv. corylina | CFBP 1159$^{PT}$ | LMG 689, NCPPB 935 | *Corylus maxima* | United States | 1939 | 2 | 2 |
| | CFBP 1846 | | *Corylus avellana* | France | 1975 | 10 | 2 |
| | CFBP 1847 | | *Corylus avellana* | Algeria | 1977 | 11 | |
| | CFBP 1848 | | *Corylus avellana* | United Kingdom | 1977 | 12 | 2 |
| | CFBP 2565 | | *Corylus avellana* | France | 1985 | 17 | 2 |
| | CFBP 5956 | | *Corylus avellana* | France | 1979 | 2 | 2 |
| | CFBP 6101 | | *Corylus avellana* | France | 1979 | 2 | 2 |
| | CFBP 6600 | | *Corylus avellana* | France | 1977 | 40 | 2 |
| *X. arboricola* pv. juglandis | CFBP 176 | | *Juglans regia* | France | 1961 | 9 | |
| | CFBP 2528$^{Te}$ | LMG 747, NCPPB 411 | *Juglans regia* | New Zealand | 1956 | 14 | 1 |
| | CFBP 2564[e] | | *Juglans regia* | Italy | 1985 | 16 | 1 |
| | CFBP 2568[e] | | *Juglans regia* | Italy | 1985 | 18 | 1 |
| | CFBP 2632 | | *Juglans regia* | Spain | 1984 | 19 | 1 |
| | CFBP 6557 | | *Juglans regia* | Italy | 1995 | 18 | 1 |
| | CFBP 7071 | | *Juglans* sp. | Spain | 1993 | 47 | 1 |
| | CFBP 7072 | | *Juglans* sp. | Spain | 1993 | 48 | 1 |
| | CFBP 7179[e] | 12763 | *Juglans regia* | France | 2002 | 6 | 1 |
| | CFBP 7244 | | *Juglans regia* | France | 1978 | 19 | 1 |
| | CFBP 7294[e] | 12578 | *Juglans regia* | France | 2001 | 3 | 1 |
| | CFBP 7296[e] | 12581 | *Juglans regia* | France | 2001 | 5 | 1 |
| | CFBP 8253[e] | 12710 | *Juglans regia* | France | 2002 | 8 | |
| | CFBP 7295[e] | 12580 | *Juglans regia* | France | 2001 | 4 | 1 |
| | CFBP 7297[e] | 12585 | *Juglans regia* | France | 2001 | 6 | 1 |
| | CFBP 7298[e] | 12588 | *Juglans regia* | France | 2001 | 7 | 1 |
| | CFBP 7299[e] | 12589 | *Juglans regia* | France | 2001 | 6 | 1 |
| | CFBP 7300[e] | 12709 | *Juglans regia* | France | 2002 | 6 | 1 |
| | CFBP 7301[e] | 12765 | *Juglans regia* | France | 2003 | 6 | 1 |
| | CFBP 7302[e] | 12770 | *Juglans regia* | France | 2003 | 6 | 1 |
| | CFBP 7303[e] | 12772 | *Juglans regia* | France | 2003 | 6 | 1 |
| | CFBP 7304[e] | 12780 | *Juglans regia* | France | 2003 | 6 | 1 |
| | CFBP 8254[e] | 12785 | *Juglans regia* | France | 2003 | 6 | 1 |
| *X. arboricola* pv. celebensis | CFBP 3523$^{PT}$ | LMG 677, NCPPB 1832 | *Musa acuminata* | New Zealand | 1960 | 34 | |
| | CFBP 7150 | | *Musa acuminata* | New Zealand | 1960 | 49 | |
| *X. arboricola* pv. fragariae | CFBP 3548 | PD 3164, LMG 19146. | *Fragaria* sp. | France | 1986 | 35 | |
| | CFBP 3549 | PD 3160 | *Fragaria* sp. | France | 1986 | 36 | |
| | CFBP 6762 | PD 2694 | *Fragaria* × *ananassa* | Italy | NA | 43 | |
| | CFBP 6763 | PD 2697 | *Fragaria* × *ananassa* | Italy | NA | 43 | |
| | CFBP 6770 | PD 2696, LMG 19144 | *Fragaria* × *ananassa* | Italy | 1994 | 44 | |
| | CFBP 6771$^{PT}$ | PD 2780, LMG 19145 | *Fragaria* × *ananassa* | Italy | NA | 45 | |
| | CFBP 6772 | PD 2803 | *Fragaria* × *ananassa* | Italy | NA | 46 | |

(Continued on following page)

**TABLE 1** (Continued)

| Taxonomic name[a] | CFBP no.[d] | Other collection no.[f] | Host of isolation | Geographic origin | Yr of isolation | ST | CC |
|---|---|---|---|---|---|---|---|
| *X. arboricola* pv. populi | CFBP 2113 | | *Populus* × *interamericana* | The Netherlands | 1980 | 13 | |
| | CFBP 2666 | | *Populus* × *interamericana* | France | 1983 | 20 | |
| | CFBP 2669 | | *Populus* × *canadensis* | France | 1987 | 21 | |
| | CFBP 2983 | | *Populus* × *canadensis* | Italy | 1989 | 22 | |
| | CFBP 2985 | | *Populus* × *interamericana* | Belgium | 1989 | 23 | |
| | CFBP 2986 | | *Populus* × *interamericana* | Belgium | 1989 | 24 | 5 |
| | CFBP 3004 | | *Populus* × *interamericana* | France | 1989 | 25 | 5 |
| | CFBP 3121 | | *Salix alba* | The Netherlands | 1980 | 26 | 6 |
| | CFBP 3122 | ICMP 9140 | *Salix alba* | The Netherlands | 1980 | 27 | 6 |
| | CFBP 3123$^{PT}$ | ICMP 8923, LMG 12141 | *Populus* × *canadensis* | The Netherlands | 1979 | 28 | |
| | CFBP 3124 | ICMP 9367, LMG 9713 | *Populus* × *generosa* | New Zealand | 1986 | 29 | 4 |
| | CFBP 3338 | | *Populus* × *interamericana* | France | 1991 | 30 | |
| | CFBP 3342 | | *Salix* sp. | New Zealand | 1988 | 31 | 4 |
| | CFBP 3343 | | *Populus* sp. | New Zealand | 1988 | 32 | |
| | CFBP 3344 | | *Salix* sp. | New Zealand | 1988 | 33 | |
| | CFBP 3839 | | *Populus deltoides* | Belgium | 1984 | 23 | |
| *X. arboricola* pv. arracaciae[b] | CFBP 7403 | IBSBF 946 | *Arracacia xanthorrhiza* | Brazil | 1992 | 52 | 7 |
| | CFBP 7404 | IBSBF 1198 | *Arracacia xanthorrhiza* | Brazil | 1995 | 52 | 7 |
| | CFBP 7405 | IBSBF 1199 | *Arracacia xanthorrhiza* | Brazil | 1995 | 52 | 7 |
| | CFBP 7406 | IBSBF 1666 | *Arracacia xanthorrhiza* | Brazil | 2001 | 53 | 7 |
| | CFBP 7407$^{PT}$ | NCPPB 2436 | *Arracacia xanthorrhiza* | Brazil | 1969 | 52 | 7 |
| *X. arboricola* pv. guizotiae[b] | CFBP 7408$^{PT}$ | NCPPB 1932 | *Guizotia abyssinica* | Ethiopia | <1966 | 54 | |
| | CFBP 7409 | NCPPB 1933 | *Guizotia abyssinica* | Ethiopia | <1966 | 54 | |
| *X. arboricola* pv. zantedeschiae[b] | CFBP 7410$^{PT}$ | NCPPB 2978 | *Zantedeschia aethiopica* | South Africa | 1967 | 55 | |
| | CFBP 7411 | NCPPB 2099 | *Zantadeschia aethiopica* | South Africa | 1966 | 55 | |
| | CFBP 7412 | NCPPB 4326 | *Zantedeschia aethiopica* | Taiwan, PRC | 2002 | 56 | |
| *X. arboricola*[c] | CFBP 7152 | LMG 5402, ICMP 3279 | *Euphorbia pulcherrima* | New Zealand | 1972 | 50 | |
| *X. arboricola*[c] | CFBP 7154 | | *Euphorbia pulcherrima* | New Zealand | 1972 | 51 | |
| *X. arboricola*[c] | CFBP 7278 | LMG 8676, ICMP 7180 | *Euphorbia pulcherrima* | New Zealand | 1980 | 51 | |
| *X. arboricola* | CFBP 1022 | | *Juglans regia* | France | 1967 | 1 | |
| *X. arboricola* | CFBP 4021 | ICMP 8452 | *Magnolia* sp. | New Zealand | 1983 | 38 | |
| *X. arboricola* | CFBP 4023 | ICMP 8457 | *Magnolia stellata* | New Zealand | 1983 | 39 | |
| *X. arboricola* | CFBP 6683 | | *Allium cepa* | Cuba | NA | 42 | |
| *X. arboricola* | CFBP 7413 | NCPPB 3200 | *Chrysanthemum morifolium* | UK ex Kenya | 1979 | 57 | |
| *X. arboricola* | CFBP 7414 | NCPPB 3218 | *Syzygium aromaticum* | Indonesia | 1980 | 58 | |
| *X. arboricola* | CFBP 7415 | NCPPB 2856 | *Chrysanthemum morifolium* | The Netherlands | 1975 | 59 | |
| *X. arboricola* | CFBP 7416 | NCPPB 2864 | *Chrysanthemum morifolium* | The Netherlands | 1975 | 59 | |
| *X. arboricola* | CFBP 7417 | NCPPB 2865 | *Chrysanthemum morifolium* | The Netherlands | 1975 | 60 | |
| *X. arboricola* | CFBP 7418 | NCPPB 2866 | *Chrysanthemum morifolium* | Netherlands | 1975 | 60 | |
| *X. arboricola* | CFBP 7419 | NCPPB 1826 | *Prunus domestica* | UK | 1966 | 61 | |

[a] Taxonomic name as suggested by this study.
[b] Comb. nov.; strains formerly classified in *X. campestris* (Approved Lists 1980 [105]).
[c] Strain formerly classified as *X. campestris* pv. poinsettiicola type C (19).
[d] CFBP, CIRM-CFBP International Center for Microbial Resources—French Collection for Plant-Associated Bacteria, Angers, France. PT, pathotype strain.
[e] Strain of *X. arboricola* pv. juglandis used in the pathogenicity assays.
[f] ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; LMG, BCCM/LMG Bacteria Collection, University of Ghent, Ghent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, York, United Kingdom; ATCC, American Type Culture Collection, Manassas, VA; PD, Culture Collection of Plant Pathogenic Bacteria, Plant Protection Service, Wageningen, The Netherlands. IBSBF, Culture Collection of Phytopathogenic Bacteria, Instituto Biologico, Campinas, SP, Brazil; no collection abbreviation, bacterial collection, UMR1345 IRHS, Beaucouzé, France.
[g] NA, information not available.

**MLST analysis.** MLST analysis was conducted using BioNumerics software and the MLST plug-in (Applied Maths, Sint Maartens-Latem, Belgium). For each locus, an allelic value was given to each unique sequence. Allelic profiles or sequence types (STs) were defined as unique combinations of seven allele numbers. STs were grouped in the same clonal complex if at least five out of seven alleles at MLST loci were identical. Comparison of STs was performed using a minimum spanning tree (MST).

**Recombination analysis.** The relative contributions of recombination and mutation to the polymorphism were estimated by the method described by Feil et al. (46). Briefly, the method focuses on the initial stages of diversification to limit homoplasy bias, and allelic comparisons are therefore made only between STs belonging to the same clonal complex. This analysis provides a lower estimation of the ratio of recombination to mutation ($r/m$), since single nucleotide changes are considered to

**TABLE 2** Primers for protein-coding-gene amplification and sequencing and consensus sequences used for trimming

| Locus | Primer name[a] | $T_a$ (°C)[e] | Sequence (5′–3′) | Size of amplicon (bp) | Consensus sequence used for trimming |
|---|---|---|---|---|---|
| *atpD* | P-X-ATPD-F[b] | 60 | GGGCAAGATCGTTCAGAT | 868 | GAAGTGCCR |
| | P-X-ATPD-R[b] | | GCTCTTGGTCGAGGTGAT | | GCRGTSGGY |
| *dnaK* | P-X-DNAK-F[b] | 60 | GGTATTGACCTCGGCACCAC | 1,034 | RAKAACACY |
| | P-X-DNAK-R[b] | | ACCTTCGGCATACGGGTCT | | YTGGTCAAG |
| *efp* | P-X-EFP-F[b] | 62 | TCATCACCGAGACCGAATA | 445 | TAYCGCTTY |
| | P-X-EFP-R[b] | | TCCTGGTTGACGAACAGC | | CCSGCVACN |
| *fyuA* | emifyuA3F[c] | 62 | ACCATCGACATGGACTGGACC | 963 | GATTGCTGC |
| | emifyuA4R[c] | | GTCGCCGAACAGGTTCACC | | ATCGGCACM |
| *glnA* | P-X-GLNA-F[b] | 60 | ATCAAGGACAACAAGGTCG | 1,094 | GAYCCGGCC |
| | P-X-GLNA-R[b] | | GCGGTGAAGGTCAGGTAG | | GGYACCAAC |
| *glnA* | GlnA-F2 | 60 | TGTCCAGCAGCACATCACC | 1,011 | |
| | GlnA-R5 | | ATCGGGGAAGCGCATTTCGAT | | |
| *gyrB* | X-gyrB1F[c] | | ACGAGTACAACCCGGACAA | 904 | CACATCCGB |
| | X-gyrB1R[c] | | CCCATCARGGTGCTGAAGAT | | GCCGARCAG |
| *rpoD* | emirpo11F[d] | 62 | ATGGCCAACGAACGTCCTGC | 1,313 | GAAATGGGY |
| | emirpo13R[d] | | AACTTGTAACCGCGACGGTATTCG | | TTCATYCGY |
| *rpoD* | rpoDX-SoF4 | 60 | GGAGCAGATCGAAGACATCATCAGC | 951 | |
| | rpoDX-SoR6 | | CATCTCGATCGAGCCCTGC | | |

[a] R, reverse primer; F, forward primer.
[b] According to Boudon et al. (66).
[c] According to Young et al. (4).
[d] According to Fargier et al. (27).
[e] $T_a$, annealing temperature for PCR.

result from mutation and multiple nucleotide changes to result from recombinational imports.

Detection of potential recombinant sequences and identification of likely parental sequences were carried out using a set of seven nonparametric detection methods implemented in RDP (Recombination Detection Program) version 3.38 (47): RDP (48), Geneconv (49), MaxChi (50), Chimaera (51), BootScan (52), SiScan (53), and 3Seq (54). The analysis was performed with default settings for the different detection methods, and the Bonferroni-corrected *P* value cutoff was set at 0.05. Recombination events were accepted when they were detected with at least three out of the seven detection methods. Split networks were constructed with SplitsTree4 V4.6 (55) software (available from http://www.splitstree.org), using the Neighbor-Net algorithm. The splits network method provides a more accurate representation of the data, since conflicting phylogenetic signals that may result from poor data or recombination events are represented by a network structure.

**Pathogenicity tests.** Pathogenicity tests were conducted on *Zantedeschia aethiopica*, *Euphorbia pulcherrima*, and *Juglans regia.* Leaves of *Z. aethiopica* were infiltrated with a bacterial suspension at $0.7 \times 10^6$ to $1.7 \times 10^6$ CFU/ml. Both sides of leaves of *E. pulcherrima* were wiped with absorbent paper soaked in a bacterial suspension at $0.7 \times 10^7$ to $1.2 \times 10^7$ CFU/ml. Infiltrations were performed in duplicate on two different plants. Sterile water was used as a negative control. The plants were grown in a climatic chamber under the following conditions: 28°C for 16 h with daylight, 25°C for 8 h in the dark, and 95% relative humidity. The appearance of symptoms was recorded 3, 7, and 10 days after inoculation. Five-year-old trees from *J. regia* cv. Fernor and cv. Chandler were inoculated in March 2007 as described by Hajri et al. (56). Eight trees per strain and three inoculation points per tree were used. Sterile water and the nonpathogenic strain CFBP 1022 were used as negative controls. Evolution of symptoms was recorded 3, 4, and 8 months after inoculation.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the partial sequences used in this study are as follows: for *atpD*, KP669177 to KP669273; for *dnaK*, KP669274 to KP669370; for *efp*, KP669371 to KP669467; for *fyuA*, KP669468 to KP669564; for *glnA*, KP669565 to KP669661; for *gyrB*, KP669662 to KP669918; and for *rpoD*, KP669919 to KP670175. Allele sequences have been deposited at the PAMDB database (7).

## RESULTS

**High levels of nucleotide and allelic diversities within the *X. arboricola* species.** Considering the seven loci, including six housekeeping genes (*atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, and *rpoD*) and one coding for a transmembrane protein (*fyuA*), 4,620 nucleotides (nt) were sequenced for 97 strains of the core collection. These strains are representative of the known diversity of *X. arboricola* species, including pathotype strains currently classified in the species. To validate the choice of the seven loci as appropriate phylogenetic markers for *X. arboricola*, descriptive statistics on nucleotide and allelic diversities were calculated for each locus and for the concatenated data set (Table 3). Insertions/deletions were found only at the *rpoD* locus; they always corresponded to 3 bp or multiples of 3 bp and thus did not modify the reading frame (data not shown). The number of haplotypes ranged from 30 (*efp*) to 41 (*rpoD*), with haplotype diversity (Hd values) ranging from 0.89 (*fyuA*) to 0.946 (*rpoD*). The nonsynonymous- to synonymous-

**TABLE 3** Descriptive statistics for polymorphism at the seven loci among 97 strains of *X. arboricola*

| Locus | Length (nt) | GC% | $S^a$ | Hap$^b$ | Hd$^c$ | $\theta\pi^d$ | $\theta^e$ | Tajima's $D^f$ | $dN^g$ | $dS^h$ | $dN/dS$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *atpD* | 750 | 64.1 | 77 | 37 | 0.934 | 0.01381 | 0.01995 | −1.00444 | 0.00090 | 0.051630 | 0.017 |
| *dnaK* | 759 | 62.3 | 90 | 36 | 0.911 | 0.01514 | 0.02304 | −1.12679 | 0.00052 | 0.06104 | 0.008 |
| *efp* | 339 | 64.9 | 28 | 30 | 0.9 | 0.00961 | 0.01605 | −1.21563 | 0.00143 | 0.03742 | 0.037 |
| *fyuA* | 753 | 64.9 | 90 | 35 | 0.89 | 0.01929 | 0.02322 | −0.55655 | 0.00507 | 0.06189 | 0.079 |
| *glnA* | 675 | 64.4 | 50 | 35 | 0.933 | 0.00894 | 0.01439 | −1.20758 | 0.00054 | 0.03548 | 0.015 |
| *gyrB* | 735 | 67.5 | 89 | 40 | 0.902 | 0.01773 | 0.02353 | −0.78361 | 0.00179 | 0.0622 | 0.028 |
| *rpoD* | 609 | 64.8 | 70 | 41 | 0.946 | 0.01681 | 0.02244 | −0.81633 | 0.00143 | 0.06283 | 0.022 |
| Concat$^i$ | 4,620 | 64.7 | 494 | 61 | 0.973 | 0.01492 | 0.02079 | −0.9601 | 0.00171 | 0.05485 | 0.03 |

[a] S, number of polymorphic sites.
[b] Hap, number of haplotypes.
[c] Hd, measurement of haplotype diversity.
[d] $\theta\pi$, Tajima's estimate of nucleotide diversity per site.
[e] $\theta w$, Watterson's estimate of the nucleotide diversity from *S* per site.
[f] Tajima's *D*, neutrality test of Tajima (36) calculated using the total number of segregating sites; all values are not significant ($P > 0.1$).
[g] *dN*, average number of pairwise differences at nonsynonymous sites.
[h] *dS*, average number of pairwise differences at synonymous sites.
[i] Concat, data representing the results obtained using a maximum-likelihood tree determined with the concatenated sequences of the seven loci.

substitution rate ratios (*dN*/*dS*) were all far less than one, which is consistent with strong selection against amino acid changes acting on housekeeping genes. The highest *dN*/*dS* ratio (0.079) was obtained for *fyuA*, which can be explained by the fact that this gene codes for a transmembrane protein and may therefore be less constrained than the housekeeping genes. The lowest *dN*/*dS* ratio (0.008) was observed for *dnaK*, which codes for a chaperone protein involved in chromosomal DNA replication. Thus, all the loci appeared to be under purifying selection, as expected for genes chosen for MLSA studies. The values for the neutrality tests of Tajima (36) were not significant, and the hypothesis of neutrality could not be rejected. The same result was obtained with the neutrality tests of Fu and Li (Fu and Li's $D^*$ and Fu and Li's $F^*$) (reference 37 and data not shown). Even if not significant, the negative tendency of Tajima's *D* values was in agreement with the purifying selection detected with the *dN*/*dS* selection test.

**Most pathovars form monophyletic clades within *X. arboricola* based on MLSA.** A clear clustering of the strains belonging to the same pathovar was seen on the phylogenetic tree based on concatenated sequences (Fig. 1). All strains of the pathovars pruni, corylina, populi, guizotiae, and arracaciae formed monophyletic groups supported by high bootstrap values (>82%). Strains of pathovar juglandis also clustered in one monophyletic group, which is, however, weakly supported by bootstrap analysis (55%), due to two divergent strains, CFBP 176 and CFBP 8253, that bifurcated at the base of the cluster. The 21 remaining strains of pathovar juglandis were grouped in a monophyletic cluster supported by a bootstrap value of 99%. Interestingly, the three most economically significant pathovars (pruni, corylina, and juglandis) were the most genetically related and form a monophyletic cluster supported by a bootstrap value of 65%. The bootstrap value reached 98% when strains CFBP 176 and CFBP 8253 were omitted (data not shown). The strains of pathovar populi formed a monophyletic cluster and were the most genetically distant from the other strains of the species *X. arboricola*, with a mean genetic distance of 2.6% ± 0.4%. The two strains of pathovar celebensis did not cluster together, nor did the seven strains of pathovar fragariae. The latter group were scattered on the phylogenetic tree in two clusters and three isolated strains. Within the three strains of pathovar zantedeschiae, the two strains from South Africa were identical and did not cluster with the strain from Taiwan. Three strains classified as *X. campestris* pv. poinsetticola type C were included in this study. All of them fit in the *X. arboricola* cluster. Two of them, CFBP 7154 and CFBP 7278, were identical at the seven loci and did not cluster with the third strain, which is distant from them (Fig. 1).

The strain CFBP 1022, isolated from *J. regia*, clustered far away from the strains of pathovar juglandis. The pathogenicity test demonstrated that the strain was not pathogenic on this host (see Fig. 5) (56). A similar result was found for the strain CFBP 7419, isolated from *Prunus domestica*, which did not cluster with the strains of pathovar pruni. This strain was originally deposited at the National Collection of Plant Pathogenic Bacteria (NCPPB) (York, United Kingdom) (NCPPB 1826) by G. E. Jones as being nonvirulent on plum fruitlets and without pathovar assignment. Nine strains included in this study were previously poorly characterized: the main associated data were the host of isolation (*Magnolia* spp., *Allium cepa*, and *Chrysanthemum morifolium*), and no information was available about their pathogenicity. These strains never fell in the clusters formed by the pathogenic strains and were poorly related to them.

This clear correspondence between phylogenetic clustering and pathovar classification was not supported by phylogenetic trees based on individual loci (see Fig. S1 in the supplemental material). An SH test was performed on ML trees derived from single loci and from the concatenated data set. Among all the combinations tested (Table 4), no tree topologies were significantly congruent ($P < 0.05$) with each other. Thus, no gene recalled the same phylogenetic history and no locus had the same evolutionary history as the one deduced from the concatenated data set. These observed incongruences might be explained by a high level of recombination that shuffles the phylogenetic signal or by the fact that each individual locus does not harbor enough phylogenetic information. It should be noted that all the phylogenetic trees (based on a single locus and multiple loci) were poorly resolved, since they exhibited low bootstrap values at deep and intermediate branches (Fig. 1; see Fig. S1 in the supplemental material).

**Phylogenetic identification of *X. arboricola* pathovars based on *gyrB* and *rpoD* sequences.** In order to propose a reduced MLSA scheme to identify the pathovars of *X. arboricola*, a neigh-

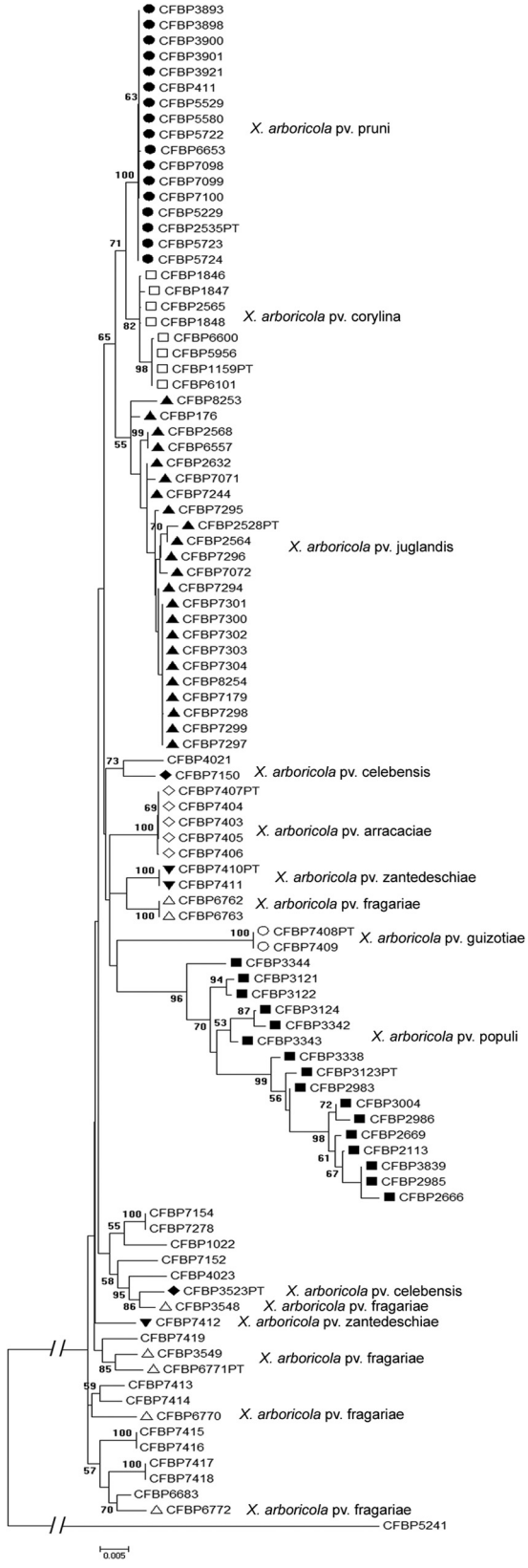


**FIG 1** Maximum-likelihood tree of 97 *X. arboricola* strains based on the concatenated set of seven partial sequences (*atpD*, *dnaK*, *efp*, *fyuA*, *glnA*, *gyrB*, and *rpoD*), representing a total of 4,620 bp, constructed with PhyML. The tree was rooted with *X. campestris* pv. campestris CFBP 5241. The confidence of the nodes was estimated with 1,000 bootstrap replicates. Bootstrap values under 50 are not shown. The scale bar indicates the number of nucleotide substitutions per site. Black circle, pathovar pruni; white square, pathovar corylina; black triangle, pathovar juglandis; black diamond, pathovar celebensis; white diamond, pathovar arracaciae; black inverted triangle, pathovar zantedeschiae; white triangle, pathovar fragariae; white circle, pathovar guizotiae; black square, pathovar populi.

bor-joining phylogenetic tree based on the concatenation of *gyrB* and *rpoD* sequences was analyzed (see Fig. S2A in the supplemental material). The *gyrB* gene was chosen because it has been used in previous studies on *Xanthomonas* diversity (4, 25, 27, 28, 57) and is becoming the reference for species phylogenetic identification within the genus. The *rpoD* gene was selected based on its high haplotype diversity measure in order to increase the discrimination between isolates (Table 3). These two genes are also used by CIRM-CFBP (http://www6.inra.fr/cirm_eng/CFBP-Plant-Associated-Bacteria) for authentication of the *Xanthomonas* resources at the species level. Using this simplified approach on the core collection, all strains of the pathovars pruni, populi, and arracaciae still formed independent monophyletic clusters (see Fig. S2A in the supplemental material). The reduced MLSA scheme is thus robust enough to identify these pathovars. The eight strains of pathovar corylina were split into two distant groups. These two groups were already identified in the complete MLSA scheme as two closely related subgroups forming a monophyletic group. For pathovar corylina, it is thus recommended to use at least two reference strains from the two clusters for phylogenetic identification, and we suggest using the pathotype strain CFBP 1159 and strain CFBP 2565. Strains of pathovar juglandis did not form a monophyletic cluster based on the two genes. Most strains of this pathovar clustered in a strongly supported monophyletic group (91% bootstrap value) together with strains of the pathovar pruni and one of the two subgroups of pathovar corylina. Within this group, strains of pathovar juglandis did not form a monophyletic subgroup. The two remaining strains, including the pathotype strain CFBP 2528, were scattered in the tree. As a consequence, strains of pathovar juglandis cannot be identified with this reduced MLSA scheme. The reduced scheme is useful to discriminate the quarantine pathovars pruni and corylina from the other strains, which cannot be achieved using only *gyrB* sequences, since strains from the pathovars juglandis, pruni, and corylina share the same *gyrB* allele (see Fig. S1 and Table S2 in the supplemental material).

Furthermore, this approach was used to type the collection of *X. arboricola* strains from CIRM-CFBP (i.e., 257 strains, including the core collection). Compared to the core collection from this study, the additional strains represented 71 strains of pathovar pruni, 48 strains of pathovar corylina, 15 strains of pathovar juglandis, 24 strains of pathovar populi, and 2 strains of *X. campestris* pv. poinsettiicola type C (see Table S1 in the supplemental material). No additional clusters were revealed on the phylogenetic tree based on the concatenation of *gyrB* and *rpoD* for this data set (see Fig. S2B in the supplemental material) compared to the one using the core collection. In addition, since strains of each pathovar clustered with their corresponding reference strains from the core collection, it can be concluded that the core collection represents the pathovar diversity within the species *X. arboricola* based on the current CIRM-CFBP collection.

**Interspecies recombination was detected at the *rpoD* and *atpD* loci.** Analysis of phylogenetic trees based on single loci re-

**TABLE 4** *P* values determined using the Shimodaira-Hasegawa test on tree topologies run on each of the maximum-likelihood trees based on the seven loci and on the concatenated sequences

| Locus | *P* value[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *atpD* | *dnaK* | *efp* | *fyuA* | *glnA* | *gyrB* | *rpoD* | Concat[b] |
| *atpD* | | 0.000 | 0.002 (0.011) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| *dnaK* | 0.000 | | 0.001 (0.002) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| *efp* | 0.000 | 0.000 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| *fyuA* | 0.000 | 0.000 | 0.000 | | 0.000 | 0.000 | 0.000 | 0.000 |
| *glnA* | 0.000 | 0.000 | 0.000 (0.001) | 0.000 | | 0.000 | 0.000 | 0.000 |
| *gyrB* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 | 0.000 |
| *rpoD* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 |
| Concat | 0.016 (0.048) | 0.035 (0.049) | 0.016 (0.026) | 0.003 (0.004) | 0.028 (0.077) | 0.003 (0.001) | 0.002 (0.000) | |

[a] The values in parentheses correspond to SH tests performed on a data set excluding CFBP 7408 and CFBP 7409.
[b] Concat, data representing the results obtained using a maximum-likelihood tree determined with the concatenated sequences of the seven loci.

vealed strains with abnormally long branches (see Fig. S1 in the supplemental material). The first example is the case of strains CFBP 7408 and CFPB 7409 of pathovar guizotiae in the *atpD* phylogenetic tree. These strains were more distant from all other *X. arboricola* strains than from the out-group strain (*X. campestris* CFBP 5241). This topology could be explained by interspecies recombination. A phylogenetic tree was constructed with the *atpD* partial sequences from strains representing the whole genus diversity (Fig. 2) to identify the origin of the allele. This tree clearly showed that the *atpD* allele from CFBP 7408 and CFBP 7409 is genetically distant from those of *X. arboricola* and more closely related to *atpD* alleles from other *Xanthomonas* species. The donor of this *atpD* allele could not be identified, since the allele of pathovar guizotiae formed a distinct branch comparable to the other *Xanthomonas* sp. branches. A similar situation was observed for the *rpoD* alleles of strains from *X. arboricola* pv. populi. The common ancestor of pathovar populi strains might have acquired its *rpoD* allele outside *X. arboricola*. Again, the donor of this allele could not be identified in our data set representing all of the described *Xanthomonas* species (data not shown).

**Economically significant pathovars are clonal complexes.** The sequence data set was analyzed using MLST (9). Unlike in MLSA, the number of nucleotide differences between alleles is not taken into account in MLST, since alleles may evolve by recombination and acquire several nucleotide differences in a single event. This approach is appropriate for microevolution studies and is widely used to identify hypervirulent lineages in clinical bacteriology. These epidemic populations are identified as clonal complexes made up of closely related STs (typically, groups of strains with six or five alleles out of seven in common). Sixty-one STs and seven clonal complexes were identified among the 97 *X. arboricola* strains (Table 1; see Tables S2 and S3 in the supplemental material). A minimum spanning tree of MLST data was generated to illustrate the presence of numerous singleton STs and the relationships between STs within the clonal complexes (Fig. 3). Clonal complexes were detected in the pathovars pruni, corylina, juglandis, arracaciae, and populi. These clonal complexes always encompassed strains from a single pathovar. The 17 strains of pathovar pruni were allocated to a single clonal complex (CC3) composed of a high-frequency ST (ST37) shared by 12 strains isolated on three continents between 1953 and 2006 and two single locus variants. Within pathovar corylina, the eight strains (isolated in the United State, France, and the United Kingdom from 1939 to 1985), except the strain isolated in Algeria, were included in CC2, which encompassed five STs. Within the pathovar juglandis, 21 strains representing 11 STs were grouped in a single CC (CC1) and two strains remained outside the CC. The five strains of the pathovar arracaciae isolated in Brazil over a period of 30 years were grouped in CC7. In contrast, among the 16 strains of pathovar populi, 10 appeared as singletons. The six remaining strains were split into three pairs of closely related STs, corresponding to CC4, CC5, and CC6. No clonal complexes were identified in pathovar fragariae despite the use of seven strains isolated in Italy and France on which the original description of the pathovar was based (20). These data indicate that the three major pathovars responsible for important economic losses, pathovars pruni, corylina, and juglandis, correspond to pandemic lineages able to maintain themselves over decades and to spread over continents. Among them, pathovar pruni is almost monomorphic and pathovar juglandis is the most polymorphic. In contrast, other pathovars, like pathovars fragariae and populi, are composed of distantly related strains, even if the collection included several strains isolated from the same country at the same period.

**Contrasting contributions of recombination and mutation in *X. arboricola* pathovars.** We attempted to estimate the role of recombination in the diversification of strains using the method suggested by Guttman and Dykhuizen (58) and described by Feil et al. (46). The principle of this method is to focus on variations between STs within clonal complexes to avoid homoplasy bias. Very different patterns of strain diversification were found between pathovars. Within CC3 (pathovar pruni) and CC7 (pathovar arracaciae), allelic differences between typical clonal alleles and variant alleles were only single nucleotide changes and thus are considered to have been brought about by mutation. In contrast, in each of the three CCs (CC4, CC5, and CC6) of pathovar populi, allelic differences always corresponded to multiple nucleotide changes and were thus considered recombinational imports. Within pathovars juglandis and corylina, the estimated ratios of recombination to mutation ($r/m$) were 4.5:1 and 0.25:1, respectively. For the whole data set, the estimated $r/m$ was 1.5:1 and the estimated per site $r/m$ parameter was 10.2, which means that a site would have 10 times more chance to change due to recombination than to change by mutation. Recombination events were seen at all loci.

The RDP package was used to detect recombination events in the sequence data set. Recombination events were accepted if they were recognized by at least three detection methods out of seven.

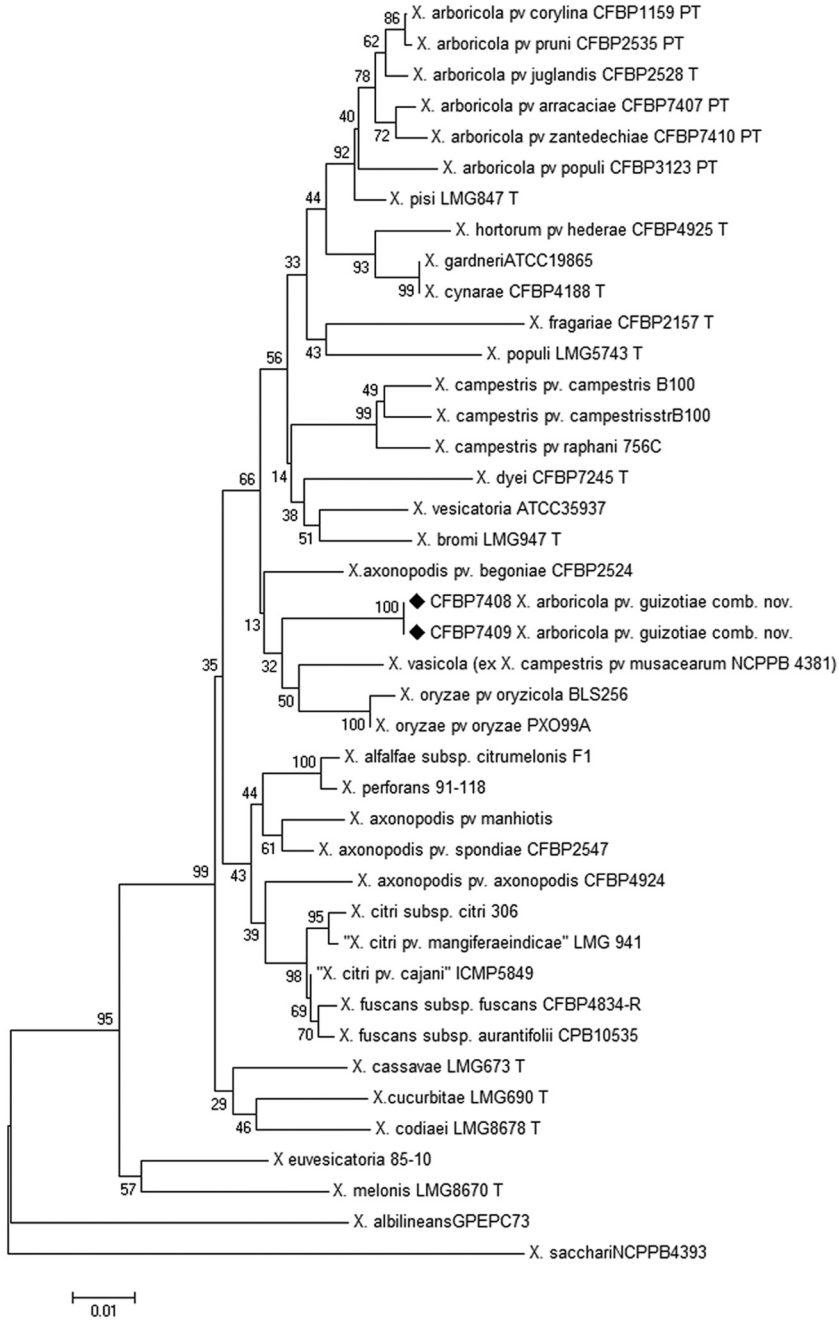


**FIG 2** NJ tree of 41 strains representative of 25 species described in the genus *Xanthomonas* based on partial nucleotide sequences of *atpD*. The confidence of the nodes was estimated with 1,000 bootstrap replicates. The scale bar indicates the number of nucleotide substitutions per site. Black diamonds highlight the *X. arboricola* strains that might have acquired their *atpD* allele through interspecies recombination.

No significant recombination event was detected in single loci. Six recombination events were detected among the 61 sequence types using the concatenated data set. These events affected strains from the pathovars populi, juglandis, pruni, guizotiae, and fragariae and strain CFBP 4021.

Incongruences between phylogenies based on individual loci, estimation of the relative contributions of recombination and mutation by the method of Feil et al. (46), and the detection of recombination events with the RDP package suggested the importance of recombination in the diversification of *X. arboricola* strains. We therefore used split graphs to represent ST relationships and to highlight conflicting signals that may be brought about by recombination (Fig. 4). Important reticulations were found on the graphs of each individual locus. On the graph based on the concatenated data set, important reticulations were observed between STs belonging to pathovars juglandis and populi, which is in accordance with the predominant role of recombination in allele diversification found in these pathovars using the method of Feil et al. (46). A striking observation is the clear separation of strains of pathovar populi from the rest of the *X.*

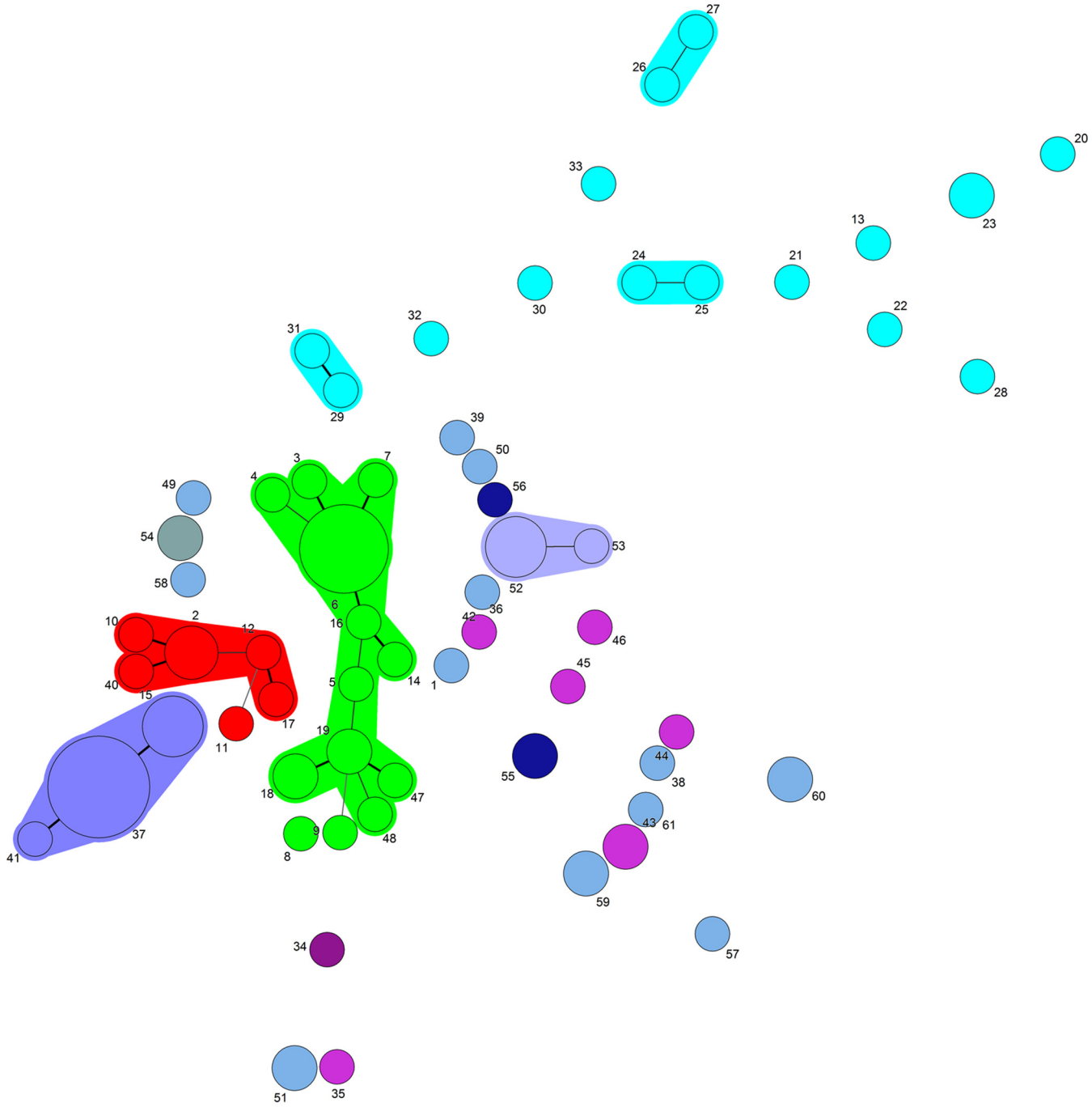


**FIG 3** Minimum spanning tree from MLST data for 97 *X. arboricola* strains representing 61 STs divided into seven CCs and 34 singleton STs constructed by BioNumerics (AppliedMaths, Belgium). Each circle represents an ST, and the frequency of the ST is correlated with the size of the circle. Clonal complexes are defined as STs that differ by one or two loci and are highlighted by a colored background. Each pathovar is represented by a different color: purple, pathovar pruni; red, pathovar corylina; green, pathovar juglandis; violet, pathovar fragariae; blue, pathovar populi; pale purple, pathovar arracaciae; gray, pathovar guizotiae; navy, pathovar zantedeschiae; faded blue, no pathovar assignment.

*arboricola* species and the differentiation of two groups within the pathovar. The first included all strains from New Zealand and two strains isolated in the Netherlands from *Salix alba*, and the second group encompassed European strains from France, Belgium, the Netherlands, and Italy isolated from *Populus*. The two strains of pathovar guizotiae (ST54) had an intermediate position between the core of *X. arboricola* and strains of pathovar populi.

**Pathogenicity tests.** The three strains CFBP 7410$^{PT}$ (pathotype strain), CFBP 7411, and CFBP 7412 from pathovar zantedeschiae were pathogenic on *Z. aethiopica*. Water-soaked necrosis surrounded by a yellow chlorotic halo appeared 3 days postinoculation (p.i.) and became larger and darker or collapsed at 7 days p.i., while no symptoms were observed on the negative control (Fig. 5). Strains CFBP 7152, CFBP 7154, and CFBP 7278, formerly classi-

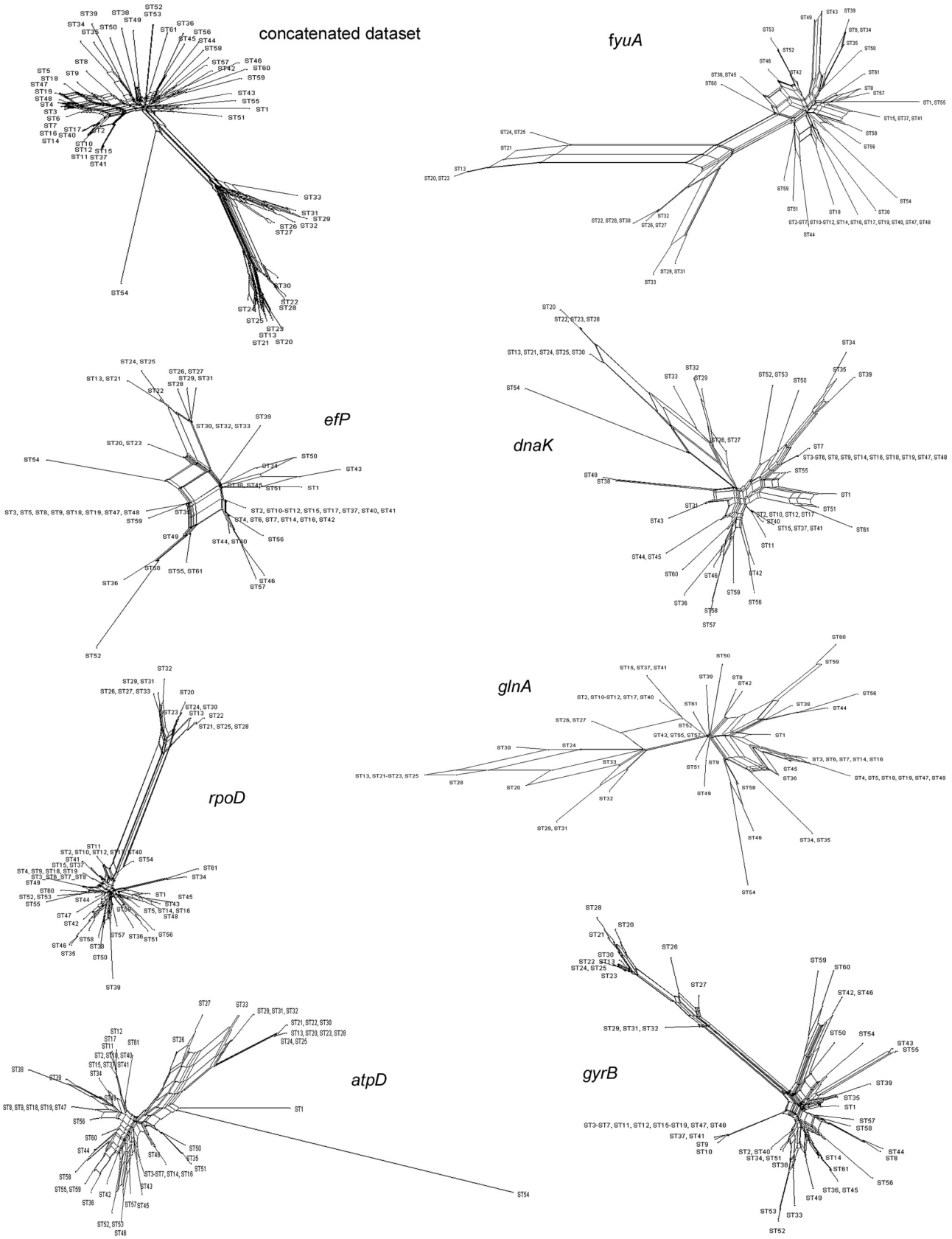


**FIG 4** Split networks for each individual locus and for the concatenated set of the seven loci. ST numbers (as defined in Table 1; see Table S2 in the supplemental material) are indicated at the branch tips.

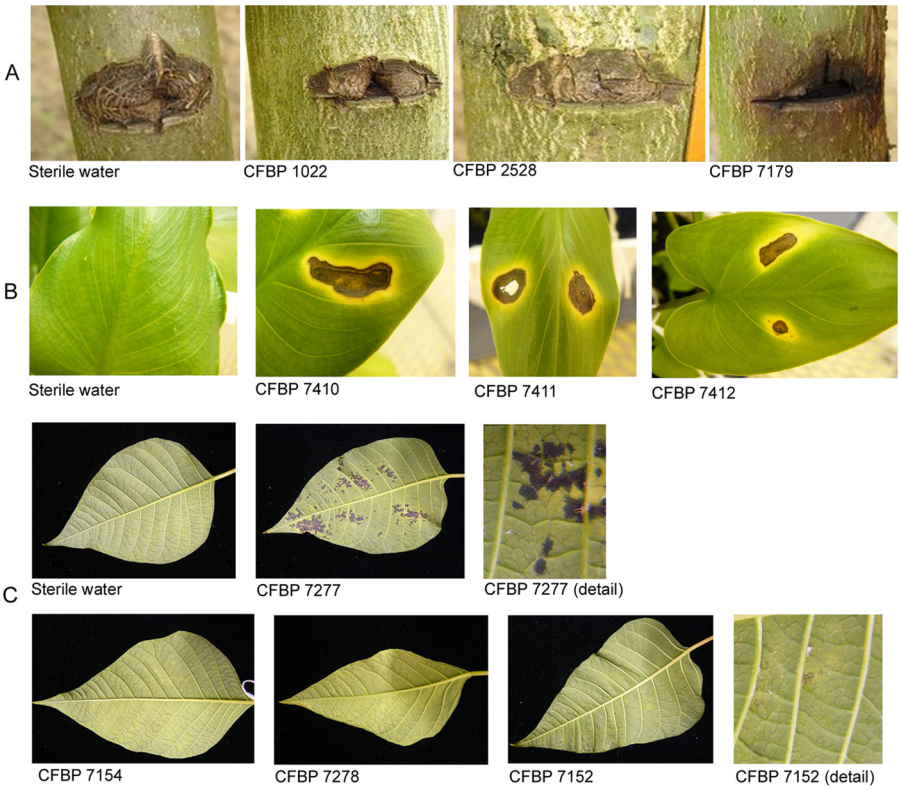


**FIG 5** Symptoms observed after artificial inoculation on *J. regia* (A), *Z. aethiopica* (B), and *E. pulcherrima* (C).

fied as *X. campestris* pv. poinsettiicola type C, were inoculated on *E. pulcherrima*. Strain CFBP 7277$^{PT}$ of *X. axonopodis* pv. poinsettiicola was used as a positive control. Only faint and rare buff-colored water-soaked lesions were observed at 10 days p.i. for strains CFBP 7152, CFBP 7154, and CFBP 7278, whereas numerous dark water-soaked spots that coalesced were observed for strain CFBP 7277$^{PT}$ (Fig. 5). Seventeen strains representing the diversity of pathovar juglandis were inoculated on the trunks of 5-year-old trees (Table 1). Typical symptoms of vertical oozing canker (VOC) (an open scar with exudate staining the bark and trunk deformations) were observed 4 months after inoculation for strains from ST3, ST4, and ST6. In contrast, healing was observed for trees inoculated with strains from ST5, ST7, ST8, ST14, ST16, and ST18.

## TAXONOMY

**Emended description of *Xanthomonas arboricola* Vauterin et al. 1995.** The description is the same as that for the genus, and biochemical discriminative characters are reported in the original description (19). The following pathovars are distinguished on the basis of phytopathogenicity specialization: *X. arboricola* pv. arracaciae comb. nov., *X. arboricola* pv. celebensis, *X. arboricola* pv. corylina, *X. arboricola* pv. fragariae, *X. arboricola* pv. guizotiae comb. nov., *X. arboricola* pv. juglandis, *X. arboricola* pv. populi, *X. arboricola* pv. pruni, and *X. arboricola* pv. zantedeschiae comb. nov. Strains of the pathovars corylina, juglandis, and pruni are classified as quarantine pests in many countries. In contrast, strains of pathovars fragariae and populi are considered saprophytic strains or opportunistic pathogens (21, 59). *X. arboricola* also includes strains that are not classified as pathovars. Pathogenic strains on *Zizyphus jujuba* (60), *Capsicum annum* (61), and *Vitis vinifera* (62) have been described without pathovar description. Strains isolated from diverse host plants (*E. pulcherrima*, *Magnolia* spp., *A. cepa*, *C. morifolium*, and *Syzygium aromaticum*) without known pathogenicity and strains from *J. regia* and *P. domestica* that are nonpathogenic on their hosts of isolation are included in the species. The type strain is CFBP 2528$^{T}$ = LMG 747$^{T}$ = NCPPB 411$^{T}$ = ICMP 35$^{T}$ = ATCC 49083$^{T}$.

**Description of *Xanthomonas arboricola* pv. arracaciae comb. nov.** *Xanthomonas arboricola* pv. arracaciae (ar.ra.ca.ci′ae N.L. fem. gen. *arracaciae* of *Arracacia*, the generic name of the plant from which the strains were isolated).

The basonym is *Xanthomonas campestris* pv. arracaciae (Pereira et al. 1971) Dye 1978 (22, 63). The description is the same as the original (22). Phylogeny based on partial sequencing of *atpD*, *dnaK*, *efp*, *fyuA*, *glnA*, *gyrB*, and *rpoD* discriminates this pathovar as a monophyletic group from other strains of *X. arboricola*. The pathotype strain is CFBP 7407$^{PT}$ = NCPPB 2436$^{PT}$ = ICMP 3158$^{PT}$ = LMG 536$^{PT}$ = Pereira SBF-913$^{PT}$.

**Description of *Xanthomonas arboricola* pv. guizotiae comb. nov.** *Xanthomonas arboricola* pv. guizotiae (gui.zo.ti′ae N.L. fem. gen. *guizotiae* of *Guizotia*, the generic name of the plant from which the strains were isolated).

The basonym is *Xanthomonas campestris* pv. guizotiae (Yirgou 1964) Dye 1978 (23, 63). The description is the same as the original (23). Phylogeny based on partial sequencing of *atpD*, *dnaK*, *efp*, *fyuA*, *glnA*, *gyrB*, and *rpoD* discriminates the original strains isolated in Ethiopia as a unique private sequence type. The pathotype strain is CFBP 7408$^{PT}$ = NCPPB 1932$^{PT}$ = ICPB XG102$^{PT}$ = ICMP 5734$^{PT}$ = LMG 731$^{PT}$ = Yirgou I-1$^{PT}$.

**Description of *Xanthomonas arboricola* pv. zantedeschiae comb. nov.** *Xanthomonas arboricola* pv. zantedeschiae (zan.te.de.schi′ae N.L. fem. gen. *zantedeschiae* of *Zantedeschia*, the generic name of the plant from which the strains were isolated).

The basonym is *Xanthomonas campestris* pv. zantedeschiae (Joubert and Truter 1972) Dye 1978 (24, 63). Partial sequencing of *atpD*, *dnaK*, *efp*, *fyuA*, *glnA*, *gyrB*, and *rpoD* discriminates two polyphyletic sequence types for strains isolated in South Africa and Taiwan, respectively. The pathotype strain is CFBP 7410$^{PT}$ = NCPPB 2978$^{PT}$ = LMG 9059$^{PT}$ = ICMP 2372$^{PT}$.

## DISCUSSION

In this study, an MLSA-MLST analysis based on the sequencing of seven protein-coding genes of a collection representative of the known diversity of *X. arboricola* has contributed valuable information about the genetic structure of the species, which is responsible for several emerging diseases that are currently on the increase worldwide (16, 17, 56, 64, 65). This is the first comprehensive study of *X. arboricola* that encompasses representative strains of all the pathovars currently classified in the species, as well as strains from diverse hosts without pathovar affiliation that were allocated to the species based on partial *gyrB* sequencing (25). Pathogenicity tests of most of the strains studied were previously published either in pathovar descriptions or in comprehensive studies (22, 23, 56, 66–68) and are supplemented with the results from this study.

The MLSA-MLST scheme revealed clustering according to pathovar classification for most pathovars. Among them, the pathovars pruni, corylina, and juglandis, which are responsible for the three most economically important diseases due to *X. arboricola*, bacterial spot of stone fruit trees, bacterial blight of hazelnut, and walnut blight (16, 69), respectively, corresponded to three different clonal complexes. These pathovars are classified as quarantine pests in many countries (https://www.ippc.int/countries/regulatedpests/). Strains of pathovar populi also clustered in a monophyletic group. However, unlike the highly pathogenic pathovars pruni, corylina, and juglandis, this monophyletic group did not correspond to a unique clonal complex, since it encompassed highly divergent genotypes. The pathovar is responsible for bark necrosis of poplar (70), a disease mostly found on 1-year-old shoots and "not considered serious" by Haworth and Spiers (59), who qualified it as an "opportunistic pathogen."

In contrast, strains that did not cluster according to their host of isolation corresponded to nonpathogenic strains (CFBP 1022 from *J. regia* and CFBP 7419 from *P. domestica*); to poorly characterized strains previously classified in *X. arboricola* without pathovar affiliation; and to strains from the pathovars fragariae, celebensis, and zantedeschiae. These strains exhibited numerous unrelated STs.

The genetic heterogeneity of strains of pv. fragariae was previously shown using multilocus sequencing and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) profiling (21). Furthermore, the authors were unable to reproduce symptoms for the 13 strains of the pathovar, although eight of them were used in the original description of the pathovar (20). Seven of these eight strains were included in this study. They also showed that most strains of the pathovar lack genes from the type III secretion system, the major pathogenicity determinant of xanthomonads. These results were confirmed in our hands (results not shown). It should also be observed that when symptoms were recorded, a long time (more than 1 month) was necessary from inoculation to symptom observation, indicating low aggressiveness, if any (20, 71). Our results support the hypothesis of Vandroemme et al. (21) that strains of pathovar fragariae represent "common plant residents," as should be the case for strains isolated from diverse hosts for which no pathovar affiliation is available.

The disease caused by pathovar celebensis is of minor incidence compared to *Xanthomonas* wilt of banana and enset due to *X. campestris* pv. musacearum. Almost no report of this disease has been documented (72), and the two strains studied were the only ones found in public culture collections.

The pathovar zantedeschiae is also a seldom-recorded pathovar (72, 73). The strain from Taiwan did not cluster with the original strains isolated in South Africa. but all of them were pathogenic on *Z. aethiopica* (Fig. 5) (73), and consequently, pathovar zantedeschiae should be considered a polyphyletic pathovar.

Thus, within *X. arboricola*, the pathogenic potential seems to be inversely correlated with the genetic diversity. The highly pathogenic strains that are responsible for pandemics in a defined host range and that are classified as a quarantine pests in many countries are clustered in clonal complexes. In contrast, strains with no, mild, or uncertain pathogenicity were represented by numerous unrelated singletons scattered in the phylogenic tree.

The numerous singletons and phylogenetic incongruences may be explained by frequent recombination (74). The *r*/*m* ratio was calculated using the method of Feil et al. (46). We found a ratio of 1.5:1, which means that recombination is slightly more frequent than mutation in *X. arboricola*. Similar values were retrieved in *X. campestris* by the same method (27) and in *X. axonopodis* with ClonalFrame (28, 75). At the nucleotidic level, the estimated per site *r*/*m* parameter in *X. arboricola* was 10.2. This ratio is 1.5 to 3 times higher than those previously estimated within other *Xanthomonas* species (27, 28) but far lower than those estimated in some human pathogens (76). A striking feature is the differences in *r*/*m* observed between pathovars. Within the pathovars pruni and arracaciae, all allelic variations were exclusively point mutations. Conversely, all allelic variants compared within pathovar populi clonal complexes resulted from recombination events. Contrasting recombination patterns between lineages were previously observed in *X. axonopodis* (28) and between phylotypes and clades of *Ralstonia solanacearum* (77). More data are needed to understand if these disparities are linked to differing ecological adaptations. Interspecies recombination at *atpD* and *rpoD* loci was also detected in our data set for strains belonging to pathovars guizotiae and populi, respectively. The occurrence of interspecies homologous recombination at housekeeping gene loci was previously revealed in other bacterial species (78, 79). Our data support a predominant role of intra- and interspecies recombination in shaping the genetic diversity of *X. arboricola*. Maynard-Smith et al. (74, 80) defined several evolutionary models for bacterial populations, from clonal to panmictic. One of these models, referred to as the "epidemic bacterial population structure," consists of a background network of recombining strains from which emerge clusters of frequently closely related genotypes. Our data suggest an epidemic population structure for *X. arboricola* with the three major pathovars as epidemic clones. To validate this hypothesis, population genetics studies, including nonpathogenic *Xanthomonas* strains (81) and strains from non-

agricultural ecosystems, are needed to better characterize the background network and to decipher the evolutionary forces responsible for the structuring and diversification of *X. arboricola*.

Despite frequent recombination, the emergence of clonal complexes and some phylogenetic signals were still detectable. The ML phylogenetic tree and the split tree based on the concatenated data set suggested a common phylogenetic origin of the three major pathovars pruni, corylina, and juglandis. The genetic relatedness between these pathovars was previously shown by repetitive extragenic palindromic PCR (rep-PCR) (71, 82), although a common origin was not clear. DNA-DNA hybridizations revealed very high levels of genomic relatedness between them compared to other *X. arboricola* strains (19). A common origin is also supported by their highly similar repertoires of T3Es (29): strains from the pathovars pruni, corylina, and juglandis had 10 T3Es in common that were not retrieved in other strains. These three pathogens are responsible for very similar symptoms on their respective hosts and share numerous biological traits (12, 69). Finally, all of them were first described in the United States at the beginning of the 20th century (11, 13, 83). MLSA data from this study, similar repertoires of virulence-associated genes, comparable symptoms, and historical reports suggest a common origin of the three major pathovars that attack stone and nut fruit trees. Comparative and population genomics will help to validate this hypothesis and to elucidate the evolutionary events that have led to the emergence of these threatening tree pathogens. Within *Pseudomonas syringae*, phylogenetic clades made up of only tree pathogens have also been observed (84, 85), suggesting a sequential adaptation, with the bacterial adaptation to tree biology being the first step that occurs before host specialization. Comparative genomics might decipher common adaptive traits of tree pathogens.

Among these three major pathogens, strains of pathovar pruni are almost monomorphic, which is characteristic of highly pathogenic strains. Isolated over a 50-year period from seven *Prunus* species and in 10 countries on three continents, these strains clustered in only one major ST (ST37) and two single locus variants. This result confirms the genetic homogeneity of this pathovar previously shown by amplified fragment length polymorphism (AFLP) and the identity of the founder genotype, which could be responsible for the pandemic observed since the 1950s (66). Strains of pathovar pruni are characterized by the presence of the ubiquitous plasmid pXap41, which is absent in other *X. arboricola* pathovars (86). Acquisition of this plasmid, which carries putative virulence-associated genes, might be responsible for the host specificity and pathoadaptation of this aggressive pest and might explain its worldwide expansion. Because MLST could distinguish only three different STs among a collection representing the diversity of the pathogen, it is recommended that a more discriminative method for detailed epidemiological studies be used, e.g., the multilocus variable number of tandem repeats analysis scheme recently developed by Cesbron et al. (87) for *X. arboricola* pathovars.

Strains of pathovar corylina were slightly more polymorphic than strains of pathovar pruni, even if the host and geographical origins of the strains were less diverse. Most of the strains studied were isolated in France in the 1970s during the first epidemic of hazelnut bacterial blight in that country (88). MLST analysis showed that all the French isolates from this outbreak were identical to the pathotype strain from Oregon, where the disease was first described (12, 83), or to single locus variants of the original strain. This result is in accordance with the observations that the first symptoms of the disease were detected in production orchards established with imported material from Oregon and that some material in the first two infected nurseries originated in the United States (88). These results support the hypothesis of an introduction of pathovar corylina in France from the United States. Contrary to results obtained by rep-PCR (67, 68), MLSA-MLST show that the pathotype strain, CFBP 1159, clusters with other pathovar corylina strains, including the strains isolated in Poland from the recent outbreak (68). Moreover, the sequences of *gyrB*, *rpoD*, and *fyuA* obtained from this strain were 100% identical to those published for the same strain preserved at NCPPB (NCPPB 935) and ICMP (ICMP 5726). Thus, the pathotype strain of *X. arboricola* pv. corylina is representative of the pathovar. Bacterial blight of hazelnut is currently spreading worldwide, and severe outbreaks were reported during the last decade (16). MLSA-MLST would be a method of choice for a comprehensive study to identify the STs responsible for these recent epidemics and to decipher the genetic relationships between older and recent isolates.

Among the three major pathovars of *X. arboricola*, pathovar juglandis is the most polymorphic, which is in accordance with previous observations (56, 89–91). Strains of the pathovar are responsible for walnut blight and VOC, a disease that appeared in French walnut orchards in the early 2000s (56, 92). MLST and pathogenicity tests showed that only a few closely related sequence types within the pathovar juglandis clonal complex are able to cause typical VOC symptoms, confirming the previous results of Hajri et al. (56) based on fluorescent AFLP (f-AFLP). The VOC strains could be differentiated from other strains of pathovar juglandis by the presence of *xopB* and the absence of *xopAH* (32). These results indicate a nested microevolution process where further specialized clones emerge from a group of strains that is already the result of a host adaptation. Remarkably, two other bacterial pathogens responsible for severe canker diseases emerged in the 2000s: *P. syringae* pv. aesculi on European horse chestnut and *P. syringae* pv. actinidiae on kiwi fruit. A pattern comparable to the one in this study is seen with epidemic clonal strains responsible for canker symptoms, whereas phylogenetically related strains can only cause leaf damage (84, 85). Comparative genomics will provide clues about the evolutionary mechanisms underlying these adaptation processes and potential common functions of pathogens of woody hosts.

These three pathovars are currently spreading, and fast and accurate diagnostic protocols are needed (16, 17, 64, 93). We showed that an MLST scheme based on seven housekeeping genes is a powerful tool to identify the pathovars and to accurately distinguish these dangerous isolates responsible for pandemics from strains of minor importance for plant health. Based on our data set, the reduced MLSA scheme using *gyrB* and *rpoD*, combined with the use of pathotype and reference strains, provided enough information to identify the two European Union quarantine pathovars pruni and corylina. Using only the *gyrB* locus to identify pathogens of stone and nut fruit trees, as suggested by Parkinson and Elphinstone (94), is not accurate, since (i) 27 strains from these three pathovars share the same *gyrB3* allele and (ii) two strains of pathovar juglandis (including the pathotype, CFBP 2528) have divergent *gyrB* alleles. For identification at the species level when only one housekeeping gene is used as a phylogenetic

marker, caution should be taken, because interspecies recombination may occur, and the use of *atpD* and *rpoD* loci alone should be avoided.

Among the strains of the heterogeneous part of *X. arboricola*, the 23 strains of pathovar populi were highly divergent. This raised the question of their affiliation with the species. Divergence of *X. arboricola* pv. populi from the rest of the species was already observed with other molecular methods (19, 82), but all of these studies used only one strain of the pathovar. We show here that all the strains of pathovar populi formed a monophyletic cluster at the root of *X. arboricola*. These strains were characterized by the important role of recombination in their diversification process, and all the strains harbored an *rpoD* allele brought about by interspecies recombination, which explains their atypical phylogenetic position in a previous study (32). It has been previously shown that MLSA provides an accurate framework for species delineation within *Xanthomonas*, and the proposed threshold, equivalent to the 70% values of DNA-DNA hybridization, was sequence similarity of 96% (4). Strains sharing similarities higher than 99% could be classified in the same species without ambiguity (4). When *rpoD* was excluded from the data set, the mean similarity between strains of pathovar populi and other *X. arboricola* strains was 97.6%, and the mean similarities within groups were 98.9% and 98.7%. The mean similarity within *X. arboricola*, including pathovar populi, was 98.5%. These values correspond to the lower boundary of the species definition. Young et al. (4) suggested that such values (96 to 99%) correspond to the subspecies threshold. A polyphasic approach is needed, which could include determination of average nucleotide identity (ANI) using genomic data, population genetics, and phenotyping to determine if strains of *X. arboricola* pv. populi should be elevated to the species or subspecies level.

Strains of *X. campestris* pv. poinsettiicola, the causal agent of bacterial leaf spot of poinsettia, were reclassified into three species: *Xanthomonas codiaei*, *X. arboricola*, and *X. axonopodis* (19). The pathotype strain, isolated in India, where the disease was first described (95), is referred to as *X. axonopodis* pv. poinsettiicola. No pathovar description or pathotype strain designation was proposed for strains assigned to *X. arboricola* (19). As a result, "*X. arboricola* pv. poinsettiicola" is an invalid name (96). Five strains reclassified in *X. arboricola* (19, 97) were included in this study. These strains, isolated in two localities in New Zealand in 1972 and 1980, were interspersed in three phylogenetic positions. This genetic heterogeneity reflected that previously observed with rep-PCR profiles (97). Bacterial leaf spot of poinsettia is an emerging disease in Europe and Asia (98). All the strains isolated from the recent outbreaks in Norway, Slovenia, Italy, Taiwan, and China were identified as *X. axonopodis* pv. poinsettiicola using appropriate methods (97, 99–102). This taxon is a quarantine pest in Europe (98). The pathogenicity tests conducted in this study with three strains of *X. arboricola* isolated from poinsettia revealed faint water-soaked symptoms on poinsettia leaves compared to those obtained with CFBP 7277, the pathotype strain of *X. axonopodis* pv. poinsettiicola (Fig. 5). This situation is similar to the one described above for pathovar fragariae (21) and to the one reported by Sawada et al. (62) for grapevine isolates classified in *X. arboricola*. These authors concluded that "the causal bacterium was a genetically heterogeneous complex of opportunistic pathogens with weak pathogenicity" and did not propose a new pathovar name for grapevine isolates. Preliminary pathogenicity tests indicated that strains of *X. campestris* pv. poinsetiicola type C have weak pathogenicity on their host of isolation. No disease reports have incriminated *X. arboricola* as the causal agent of bacterial leaf spot of poinsettia. Altogether, this restrains us from proposing a new pathovar to accommodate these strains, and we propose to rename them *X. arboricola*. Thorough ecological studies on strains with uncertain pathogenicity that focus on host specialization and population dynamics are necessary to understand their ecological significance.

It has been previously shown that the pathotype strains of *X. campestris* pv. arracaciae, *X. campestris* pv. guizotiae, and *X. campestris* pv. zantedeschiae were allocated to *X. arboricola* (25). These three pathogens are seldom-recorded pathogens, and few strains are publically available. We included strains of these pathovars available in public repositories in our study of *X. arboricola*. The five strains of pathovar arracaciae isolated in Brazil over a 30-year span clustered in a tight clonal complex clearly separated from other strains. The pathovar is the causal agent of leaf spot of arracacha (*Arracacia xanthorrhiza*), a common disease in Brazil (103). The two strains of pathovar guizotiae were isolated in Ethiopia, where the bacterial leaf spot of niger (*Guizotia abyssinica*) was first described (23). They exhibited the same genotype (ST54), which was genetically divergent from all other STs, and harbored an *atpD* haplotype brought about by interspecies recombination. These strains represented an original phylogenetic lineage within the species. Bacterial leaf spot of this oilseed crop was also described in India (23, 104), and it would be interesting to type Indian isolates to determine their genetic relatedness to the African isolates. Within pathovar zantedeschiae, the two strains that originated in South Africa, where the disease was first described (24), did not cluster with the strain that originated in Taiwan (73). Based on seven housekeeping genes and using a comprehensive collection of *X. arboricola* strains, we confirmed that strains of *X. campestris* pv. arracaciae, *X. campestris* pv. guizotiae, and *X. campestris* pv. zantedeschiae belonged to *X. arboricola*, and we propose to reclassify these three pathovars in the species as *X. arboricola* pv. arracaciae comb. nov, *X. arboricola* pv. guizotiae comb. nov., and *X. arboricola* pv. *zantedeschiae* comb. nov. *X. arboricola* Vauterin et al. 1995 is emended to include these three former *X. campestris* pathovars and strains without pathovar affiliation.

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