Type Three Effector Gene Distribution and Sequence Analysis Provide New Insights into the Pathogenicity of Plant-Pathogenic Xanthomonas arboricola

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Type Three Effector Gene Distribution and Sequence Analysis Provide New Insights into the Pathogenicity of Plant-Pathogenic *Xanthomonas arboricola*

Ahmed Hajri,[a] Joël F. Pothier,[b] Marion Fischer-Le Saux,[a] Sophie Bonneau,[a] Stéphane Poussier,[c] Tristan Boureau,[d] Brion Duffy,[b] and Charles Manceau[a]

INRA, UMR 077 PaVé, Beaucouzé, France[a]; Research Station Agroscope Changins-Wädenswil ACW, Plant Protection Division, Wädenswil, Switzerland[b]; Département de Sciences Biologiques, Agrocampus Ouest, Centre d'Angers, Institut National d'Horticulture et de Paysage, UMR 077 PaVé, Beaucouzé, France[c]; and Département de Biologie, Université d'Angers, UMR 077 PaVé, Beaucouzé, France[d]

***Xanthomonas arboricola* is a complex bacterial species which mainly attacks fruit trees and is responsible for emerging diseases in Europe. It comprises seven pathovars (*X. arboricola* pv. pruni, *X. arboricola* pv. corylina, *X. arboricola* pv. juglandis, *X. arboricola* pv. populi, *X. arboricola* pv. poinsettiicola, *X. arboricola* pv. celebensis, and *X. arboricola* pv. fragariae), each exhibiting characteristic disease symptoms and distinct host specificities. To better understand the factors underlying this ecological trait, we first assessed the phylogenetic relationships among a worldwide collection of *X. arboricola* strains by sequencing the housekeeping gene *rpoD*. This analysis revealed that strains of *X. arboricola* pathovar populi are divergent from the main *X. arboricola* cluster formed by all other strains. Then, we investigated the distribution of 53 type III effector (T3E) genes in a collection of 57 *X. arboricola* strains that are representative of the main *X. arboricola* cluster. Our results showed that T3E repertoires vary greatly between *X. arboricola* pathovars in terms of size. Indeed, *X. arboricola* pathovars pruni, corylina, and juglandis, which are responsible for economically important stone fruit and nut diseases in Europe, harbored the largest T3E repertoires, whereas pathovars poinsettiicola, celebensis, and fragariae harbored the smallest. We also identified several differences in T3E gene content between *X. arboricola* pathovars pruni, corylina, and juglandis which may account for their differing host specificities. Further, we examined the allelic diversity of eight T3E genes from *X. arboricola* pathovars. This analysis revealed very limited allelic variations at the different loci. Altogether, the data presented here provide new insights into the evolution of pathogenicity and host range of *X. arboricola* and are discussed in terms of emergence of new diseases within this bacterial species.**

Xanthomonas arboricola is a complex bacterial species mainly comprising plant-pathogenic bacteria which cause diseases on fruit trees and is responsible for emerging diseases in Europe (11, 24, 28, 53, 61, 72). It encompasses seven pathovars with different hosts, including *X. arboricola* pv. pruni (host, stone fruits), *X. arboricola* pv. corylina (hazelnut), *X. arboricola* pv. juglandis (Persian walnut), *X. arboricola* pv. populi (poplar), *X. arboricola* pv. poinsettiicola (poinsettia) (72), *X. arboricola* pv. celebensis (banana) (45), and *X. arboricola* pv. fragariae (strawberry) (27). The phylogenetic relationships within *X. arboricola* species were assessed using different methods, showing that the different pathovars formed well-defined groups in relation to their phytopathogenic specialization and that pathovars pruni, corylina, and juglandis are the most closely related (46, 53, 61, 72, 75). These three closely related *X. arboricola* pathovars are considered to be the most economically important ones, whereas the other pathovars are considered to be of less economic importance (28, 61, 72). Indeed, bacterial spot of stone fruits (*X. arboricola* pv. pruni) and bacterial blight of hazelnut (*X. arboricola* pv. corylina) are emerging diseases in several European countries and are included in the A2 alert list published by the European and Mediterranean Plant Protection Organization (EPPO) (6, 28, 60). In addition, *X. arboricola* pv. pruni is classified as a quarantine organism by the phytosanitary legislation of the European Union (EU) (5). *X. arboricola* pv. juglandis is the causal agent of walnut blight (WB), one of the most serious diseases of Persian (English) walnut in all walnut-growing areas (34). Recently, a new bacterial disease, termed vertical oozing canker (VOC), emerged in French walnut orchards, and its causal agent was identified as a distinct genetic lineage within *X. arboricola* pv. juglandis (24).

Because of their economic and regulatory status, *X. arboricola* pathovars pruni, corylina, and juglandis have already been the subject of many epidemiological and population structure studies (10, 11, 13, 24, 36, 49, 59, 60, 76). *X. arboricola* pv. pruni is characterized by very low genetic diversity, and partitioning of strains at a geographical scale has not been observed. This might be due to the extensive distribution of the same peach and Japanese plum cultivars in all areas of cultivation and also to the very limited genetic diversity of the host (11, 76). In contrast, the genetic diversity of pathovar juglandis is relevant, and clustering of strains at a geographical scale is possible. This is likely because Persian walnut cultivation is based mainly on local seedlings which have adapted to particular environments and thus enabled selection of different *X. arboricola* pv. juglandis populations (36, 59). The genetic diversity of pathovar corylina is also high, because strains isolated from *Corylus maxima* were shown to deviate genetically and pathogenically from strains isolated from *Corylus avellana* (60). Taken together, these studies underlined the role of host

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Address correspondence to Charles Manceau, charles.manceau@angers.inra.fr.

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selection in structuring the populations of these three important stone fruit and nut pathogens. However, other important aspects which may influence the overall population structure of these bacterial pathogens remain to be elucidated, and to date a comparative study based on the genomic and pathogenic features of all *X. arboricola* pathovars has not been undertaken.

As for many plant-pathogenic bacteria, host specialization is very high for bacteria belonging to *X. arboricola*. Within this species, the host range of the different pathovars is restricted to one or a few host plants, reflecting a close adaptation to the host (53, 72). Elucidation of the molecular basis of the interactions between *Xanthomonas* strains and their host plants is a fascinating question which is being accelerated by advances in genomic sequencing. Complete genome sequences are available for several *Xanthomonas* species, including *X. axonopodis*, *X. campestris*, *X. oryzae*, *X. albilineans*, *X. fuscans*, *X. vasicola*, *X. hortorum*, *X. perforans*, and *X. gardneri* (14, 29, 33, 39, 43, 47, 50, 51, 56, 66, 69, 73). Comparative genomic analyses identified several molecular mechanisms which might promote the evolution and adaptation of *Xanthomonas* strains to diverse environments and host plants, such as acquisition of virulence-associated genes by horizontal gene transfer, occurrence of accessory and mobile genetic elements (e.g., integrons, plasmids, phages, and transposons), and spontaneous mutations. Studies concerning these aspects of *X. arboricola* are lacking, and current knowledge is limited to the diversity of integrons in some of the pathovars defined within this species (8, 9, 18). Furthermore, no published genome sequences are available for *X. arboricola*, and as a consequence, little is known about the genetic basis of the differing host specificities between *X. arboricola* pathovars and the forces which led to the emergence of new diseases (e.g., VOC in France) within this species. The identification of complete repertoires of virulence-associated genes of *X. arboricola* pathovars is necessary to better understand pathogen-plant interactions, e.g., differences in host range.

Among virulence-associated genes that may account for the pathological adaptation of plant-pathogenic bacteria to their hosts, the role of type III effectors (T3Es) is the best documented to date. T3Es are delivered directly into the host cells through the type III secretion system (T3SS), a highly conserved protein secretion system whose structural components are encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes (1). Once inside the host cells, T3Es are reported to modulate host defense signaling pathways and to induce disease by interfering with host cell functions (2, 19, 20). In many plant-pathogenic bacteria, it has been established that T3Es can act as molecular double agents which betray the pathogen to plant defenses in some interactions and suppress host defense mechanisms in others, thus limiting and enlarging the pathogen's host range (2, 12, 19, 20, 22). Previous studies identified the presence of complex T3E repertoires in the genomes of several plant-pathogenic species and pathovars. These studies revealed that T3E repertoires differ between species and between strains within species and are consequently believed to be among the main determinants of the host range (19, 21, 23, 54, 57).

To further elucidate the possible role of T3Es in defining host specificity of the species *X. arboricola*, we surveyed the distribution of 53 T3E genes in a worldwide collection of strains of this species. The strains used reflected a wide range of collection dates, locations, and host plants. The phylogenetic positions of strains used in this work were obtained by sequencing the housekeeping gene *rpoD*. In order to reinforce the data we obtained using the presence/absence of T3E genes as criteria, we then sequenced some T3E genes and studied their allelic diversity in *X. arboricola* pathovars. The results of our study provide new insights into the involvement of T3E repertoires in host specificity and pathogenicity of the species *X. arboricola* and the driving forces which might contribute to the emergence of new diseases within this complex bacterial species. Our work also provides clues for functional studies aiming at understanding virulence and host specificities of *X. arboricola* pathovars.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. In total, we built up a working collection of 78 strains, including 73 strains belonging to *X. arboricola* and 5 strains belonging to *X. populi*, the latter being included for comparative purposes in our phylogenetic analysis. Strains were collected from various hosts and locations and at different times. Each pathovar of *X. arboricola* was represented by at least 10 strains, except for pathovars celebensis and poinsettiicola, for which only two strains were available in international collections, and pathovar fragariae, for which only strains studied by Janse et al. (27) available in the French collection of plant pathogenic bacteria (CFBP) were included. The genetic diversity and pathogenicity of the majority of the strains of *X. arboricola* studied have already been assessed (11, 16, 24, 27, 36, 46, 53, 59, 60, 61, 72, 75). The *X. populi* strains included representatives of the two subspecies described within this species (15). We also included *Xanthomonas* strains whose genomes were sequenced as positive and negative controls for detection of T3E genes: *X. axonopodis* pv. vesicatoria strain CFBP5618 (85-10), *X. axonopodis* pv. citri strain 306, *X. campestris* pv. campestris strains CFBP5241 (ATCC 33913) and B100, and *X. oryzae* pv. oryzae strain MAFF311018 (http://www.xanthomonas.org/t3e.html). Bacterial strains were routinely cultured on YPGA medium (7 g liter^{-1} yeast extract, 7 g liter^{-1} peptone, 7 g liter^{-1} glucose, 18 g liter^{-1} agar; pH 7.2) for 2 to 4 days at 28°C. *X. populi* strains were grown on slants of YPGA at 24°C (40). For PCRs, bacterial suspensions (3×10^8 CFU ml^{-1}) were prepared from fresh cultures and used as templates for amplification.

Phylogenetic analysis. The phylogenetic analysis of *X. arboricola* strains was performed by sequencing *rpoD* (RNA polymerase sigma 70 factor), one of the housekeeping genes commonly used in xanthomonad phylogeny (17, 23, 75). Gene fragments were amplified with primers described by Hajri et al. (23). PCR amplifications were carried out in a total volume of 25 μl containing 1× GoTaq buffer (Promega), 200 μM (each) deoxynucleoside triphosphates (dNTPs), 0.5 μM (each) primers, 0.4 U of GoTaq polymerase (final concentrations), and 5 μl of boiled bacterial cells (3×10^8 CFU ml^{-1}). The PCR cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 30 s, and a final extension step at 72°C for 7 min. PCR amplicons were then sent to the Biogenouest platform for sequencing (Nantes, France). Forward and reverse sequences were obtained using the *rpoD*-specific PCR primers. Nucleotide sequences were edited and assembled using PREGAP 4 and GAP 4 of the Staden software package (63) and then aligned using ClustalW (71). Amino acid alignments were transposed back to the nucleotide sequence level to gain a codon-based alignment (26). A BLOSUM protein weight matrix series was used to align the sequences, and a neighbor-joining tree was generated with the MEGA 4.0.2 software program (68) using the Kimura two-parameter model (30) and 1,000 bootstrap replicates. *X. populi* strains were also included in the phylogenetic analysis for comparison, and *X. campestris* pv. campestris strain CFBP5241 was used as an outgroup.

Amplification of T3E genes. In this study, we used the nomenclature and classification scheme for T3E genes in xanthomonads recently described by White et al. (74) and available in the *Xanthomonas* resource website (http://www.xanthomonas.org/t3e.html). The presence of 53 T3E genes in our bacterial collection was evaluated by PCR using two sets of

TABLE 1 Bacterial strains used in this study[a]

Xanthomonas species and pathovar	CFBP strain no.[b]	Non-CFBP strain(s)	Host	Geographic origin	Yr of isolation
X. arboricola pv. pruni	5530		*Prunus persica*	Italy	1989
X. arboricola pv. pruni	3893		*Prunus persica*	Italy	1989
X. arboricola pv. pruni	3921		*Prunus persica*	Italy	1996
X. arboricola pv. pruni	5722		*Prunus persica*	Brazil	1991
X. arboricola pv. pruni	5529	NCPPB 1607	*Prunus persica*	Australia	1964
X. arboricola pv. pruni	411	ATCC 10016, ICMP 12475	*Prunus persica*	United States	1963
X. arboricola pv. pruni	3900		*Prunus persica*	United States	1987
X. arboricola pv. pruni	3898		*Prunus domestica*	United States	1989
X. arboricola pv. pruni	5724		*Prunus amygdalus*	United States	NA[c]
X. arboricola pv. pruni	3901		*Prunus armeniaca*	United States	1987
X. arboricola pv. pruni	7098		*Prunus domestica*	Spain	2002
X. arboricola pv. pruni	7100		*Prunus dulcis*	Spain	2006
X. arboricola pv. pruni	12984		*Prunus armeniaca*	Switzerland	2005
X. arboricola pv. pruni	12972		*Prunus armeniaca*	Switzerland	2008
X. arboricola pv. pruni	5580		*Prunus japonica*	France	2000
X. arboricola pv. pruni	6653		*Prunus persica*	France	2000
X. arboricola pv. pruni	5575		*Prunus armeniaca*	France	2000
X. arboricola pv. pruni	5229		*Prunus* sp.	Argentina	1996
X. arboricola pv. pruni	3894[Pt]	NCPPB 416, ATCC 19316, LMG 852	*Prunus salicina*	New Zealand	1953
X. arboricola pv. pruni	5723		*Prunus* sp.	Uruguay	NA
X. arboricola pv. corylina	1159[Pt]	ATCC 19313, LMG 689, NCPPB 935	*Corylus maxima*	United States	1939
X. arboricola pv. corylina	1847		*Corylus avellana*	Algeria	1977
X. arboricola pv. corylina	1848		*Corylus avellana*	United Kingdom	1977
X. arboricola pv. corylina	7381	NCPPB 2896	*Corylus avellana*	United Kingdom	1976
X. arboricola pv. corylina	2565	ICMP 11956	*Corylus avellana*	France	1985
X. arboricola pv. corylina	5956		*Corylus avellana*	France	1979
X. arboricola pv. corylina	6006		*Corylus avellana*	France	1975
X. arboricola pv. corylina	6101		*Corylus avellana*	France	1979
X. arboricola pv. corylina	7384	NCPPB 3875	*Corylus avellana*	Italy	1991
X. arboricola pv. corylina	7385	NCPPB 3876	*Corylus avellana*	Italy	1991
X. arboricola pv. juglandis	2528[T]	ATCC 49083, LMG 747, NCPPB 411	*Juglans regia*	New Zealand	1956
X. arboricola pv. juglandis	2568		*Juglans regia*	Italy	1985
X. arboricola pv. juglandis	2564	ICMP 11955	*Juglans regia*	Italy	1985
X. arboricola pv. juglandis	2632	ICMP 11963	*Juglans regia*	Spain	1984
X. arboricola pv. juglandis	176		*Juglans regia*	France	1961
X. arboricola pv. juglandis	12578		*Juglans regia*	France	2001
X. arboricola pv. juglandis	12581		*Juglans regia*	France	2001
X. arboricola pv. juglandis	12775		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12680		*Juglans regia*	France	2002
X. arboricola pv. juglandis	12783		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12585*		*Juglans regia*	France	2001
X. arboricola pv. juglandis	12770*		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12590*		*Juglans regia*	France	2001
X. arboricola pv. juglandis	12772*		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12763*		*Juglans regia*	France	2002
X. arboricola pv. juglandis	12785*		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12780*		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12765*		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12768*		*Juglans regia*	France	2003
X. arboricola pv. juglandis	12784*		*Juglans regia*	France	2003
X. arboricola pv. celebensis	3523[PT]	LMG 677, NCPPB 1832, ATCC 19045	*Musa acuminata*	New Zealand	1960
X. arboricola pv. celebensis	7150	LMG 676, NCPPB 1630, ICMP 1484	*Musa acuminata*	New Zealand	1960
X. arboricola pv. poinsettiicola	7152	LMG 5402, ICMP 3279	*Euphorbia pulcherrima*	New Zealand	1972
X. arboricola pv. poinsettiicola	7278	LMG 8676, ICMP 7180	*Euphorbia pulcherrima*	New Zealand	1980
X. arboricola pv. fragariae	3548	PD 3164, LMG 19146	*Fragaria* sp.	France	1986
X. arboricola pv. fragariae	6762	PD 2694	*Fragaria* × *ananassa*	Italy	NA
X. arboricola pv. fragariae	6763	PD 2697	*Fragaria* × *ananassa*	Italy	NA
X. arboricola pv. populi	3121		*Salix alba*	Netherlands	1980
X. arboricola pv. populi	3122	ICMP 9140	*Salix alba*	Netherlands	1980
X. arboricola pv. populi	2113		*Populus* × *interamericana*	Netherlands	1980
X. arboricola pv. populi	3123[PT]	ICMP 8923, LMG 12141	*Populus* × *canadensis*	Netherlands	1979
X. arboricola pv. populi	2666		*Populus* × *interamericana*	France	1983
X. arboricola pv. populi	2669		*Populus* × *canadensis*	France	1987
X. arboricola pv. populi	3004		*Populus* × *interamericana*	France	1989
X. arboricola pv. populi	3338		*Populus* × *interamericana*	France	1991
X. arboricola pv. populi	3839		*Populus deltoides*	Belgium	1984
X. arboricola pv. populi	2986		*Populus* × *interamericana*	Belgium	1989
X. arboricola pv. populi	2985		*Populus* × *interamericana*	Belgium	1989
X. arboricola pv. populi	3124	ICMP 9367, LMG 9713	*Populus* × *generosa*	New Zealand	1986
X. arboricola pv. populi	3342		*Salix* sp.	New Zealand	1988
X. arboricola pv. populi	3343		*Populus* sp.	New Zealand	1988
X. arboricola pv. populi	3344		*Salix* sp.	New Zealand	1988
X. arboricola pv. populi	2983		*Populus* × *canadensis*	Italy	1989
X. populi	1817	ATCC 51165, ICMP 5816, LMG 5743	*Populus* × *canadensis*	France	1957
X. populi	2193	LMG 5772	*Populus trichocarpa*	Belgium	1983
X. populi	6294		*Populus* × *interamericana*	United Kingdom	1990
X. populi	6635	ICMP 9985	*Salix* × *dasyclados*	Netherlands	1988
X. populi	6558	NCPPB 3426, ICMP 7999	*Salix dasyclada*	Netherlands	1976

[a] CFBP, CIRM/CFBP Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; 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, Netherlands.

[b] T, type strain; Pt, pathotype strain. *X. arboricola* pv. juglandis strains that belong to the VOC cluster on the basis of f-AFLP analysis (24) are indicated with an asterisk.

[c] NA, not known.

primers for each gene, enabling amplification of full-length and partial T3E DNA sequences, except for the *avrBs3*, *xopZ*, *xopAM*, and *xopAD* genes, for which only partial T3E DNA sequences were amplified (Table 2). The T3E gene primers were designed using the Amplify software program, version 3.1.4. The genome of *X. arboricola* pv. pruni strain CFBP5530, which has been sequenced to draft status by Agroscope Changins-Wädenswil Research Station ACW (Switzerland), was used as a reference to design the primers. Using BLASTX analysis with default parameters (4), 21 T3E gene orthologues were identified in this genome among the 53 T3E genes studied. For these 21 T3E genes, primers were designed based on the genome of *X. arboricola* pv. pruni strain CFBP5530, which was subsequently used as a positive control for the PCRs (Table 2). For the remaining 32 T3E genes, sequences of *X. axonopodis* pv. vesicatoria strain CFBP5618, *X. axonopodis* pv. citri strain 306, *X. campestris* pv. campestris strains CFBP5241 and B100, and *X. oryzae* pv. oryzae strain MAFF311018 (http://www.xanthomonas.org/t3e.html) were aligned, and conserved regions in T3E gene sequences were identified and then used to design the primers (Table 2).

For detection of T3E genes, PCRs were carried out in a total volume of 20 μl containing 1× GoTaq buffer (Promega), 200 μM (each) dNTPs, 0.5 μM (each) primers, 0.4 U GoTaq polymerase (final concentrations), and 5 μl of boiled bacterial cells (3×10^8 CFU ml^{-1}). All PCRs were performed with the following cycling conditions: initial denaturation step at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C or 65°C (depending on the T3E genes) for 1 min, and extension at 72°C for 2 min, and a final extension step at 72°C for 10 min. Amplification products were separated on a 1.5% agarose gel in Tris-borate-EDTA (TBE) stained with ethidium bromide. Presence/absence of a band of the expected size was taken as an indication of the presence or absence of T3E genes in the genomes of the strains tested. A subset of T3E genes was replicated twice to check the reproducibility of the PCRs. We also assessed the robustness of our PCR results using the draft genome sequence of *X. arboricola* pv. pruni strain CFBP5530 and published genomes of *Xanthomonas* (http://www.xanthomonas.org/t3e.html). Thus, we were able for these strains to compare the T3E repertoires obtained by our PCR approach with the expected T3E repertoires based on the genome sequences. For each of these strains, every PCR result was confirmed by BLAST analysis (4) and the obtained T3E repertoires corresponded to the expected T3E repertoires.

Sequence analysis of T3E genes from *X. arboricola* pathovars. To determine the nucleotide sequences of T3E genes from *X. arboricola* pathovars, the PCR products of eight T3E genes (Table 3) were sequenced by the Biogenouest platform (Nantes, France) using forward and reverse PCR primers which enable amplification of full-length T3E DNA sequences (Table 2). These sequences were edited and assembled using PREGAP 4 and GAP 4 of the Staden software package (63) and then aligned using ClustalW (71). Phylogenetic trees were constructed from the nucleotide alignments by using the neighbor-joining method (55) with the Kimura two-parameter model (30). Bootstrapping was performed with 1,000 replicates. The resulting phylogenetic trees were visualized using the MEGA 4.0.2 software program (68).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this work were deposited in GenBank under accession numbers JF759825 to JF759902 for *rpoD*, JF826871 to JF826895 for *xopA*, JF826896 to JF826904 for *xopAI*, JF826905 to JF826910 for *xopB*, JF826911 to JF826916 for *xopE3*, JF826917 to JF826922 for *xopG*, JF826923 to JF826928 for *xopH*, JF826929 to JF826943 for *xopQ*, and JF826944 to JF826958 for *xopV*.

RESULTS

The *X. arboricola* species comprises monophyletic and polyphyletic pathovars. Before investigating the T3E gene distribution, we determined the phylogenetic relatedness among the strains tested by sequencing the housekeeping gene *rpoD*. The neighbor-joining tree, rooted with the orthologue *rpoD* sequence from *X. campestris* pv. campestris strain CFBP5241, is presented in Fig. 1. According to the results of this phylogenetic analysis, strains were clustered into three main groups. The first one gathered all the strains from *X. arboricola* with the exception of strains from pathovar populi, the second one contained all *X. arboricola* pv. populi strains tested that were found to be separated from the coherent main *X. arboricola* cluster formed by all other strains, and the third one corresponded to strains from *X. populi* (Fig. 1). Our *rpoD*-based tree also revealed that most of the *X. arboricola* strains fell into clusters according to their pathovar affiliation. Strains from pathovars pruni, corylina, and populi clustered, respectively, in monophyletic groups supported by high bootstrap values. Strains from pathovar juglandis fell into three clusters. In contrast, strains from pathovars fragariae, celebensis, and poinsettiicola exhibited divergent *rpoD* sequences and did not form clusters (Fig. 1).

Among the monophyletic pathovars, strains of *X. arboricola* pv. pruni were the most homogeneous ones, exhibiting completely identical *rpoD* sequences, followed by strains from *X. arboricola* pv. corylina, within which only strain CFBP1159 exhibited a different allele from other strains of this pathovar (Fig. 1). In contrast, *X. arboricola* pv. populi strains, which were also gathered in a monophyletic cluster, were more heterogeneous and were scattered into different subclusters with more variable *rpoD* sequences (Fig. 1). In this case, no correlation between clustering of *X. arboricola* pv. populi strains and their geographical origins was found. In some cases, strains isolated from different countries showed the same *rpoD* sequence (i.e., CFBP2669 isolated in France and CFBP3123 isolated in the Netherlands). In other cases, strains isolated in the same country showed different *rpoD* sequences (i.e., strains CFBP3121 and CFBP2113, both isolated in the Netherlands) (Fig. 1). Finally, strains of *X. arboricola* pv. juglandis were separated into three clusters, with VOC strains forming a cluster which was clearly separated from WB strains, which were clustered into two distinct subclusters (Fig. 1).

Our phylogenetic analysis clearly revealed that *X. arboricola* pv. populi strains tested form a distinct cluster separated from all other *X. arboricola* strains tested. The divergence of *X. arboricola* pv. populi strains from the core of the species *X. arboricola* was already noticed in previous studies using DNA-DNA hybridizations, amplified fragment length polymorphism (AFLP), repetitive PCR (rep-PCR), and multilocus sequence analysis (MLSA) techniques (46, 52, 53, 72, 75). From our phylogenetic analysis, we can conclude that the studied strains of *X. arboricola* pv. populi belong to a monophyletic cluster, the position of which remains uncertain. In addition, *X. arboricola* pv. populi strains represent a group of putative nonpathogenic isolates within the species *X. arboricola*, as previously suggested by pathogenicity tests (16). For these reasons, *X. arboricola* pv. populi strains were excluded from further analysis.

The sizes of T3E repertoires vary greatly between *X. arboricola* pathovars. Before investigating the T3E gene distribution in our collection of strains, we checked that all strains of the main *X. arboricola* cluster (from pathovars pruni, corylina, juglandis, poinsettiicola, celebensis, and fragariae) have a T3SS of the Hrp2 family that is usually present in xanthomonads. This distribution analysis was performed using specific primers (Table 4) for 11 genes coding for the structural and regulatory components of the highly conserved T3SS of the Hrp2 family which is usually present in xanthomonads (1, 22). This analysis revealed that all strains tested harbored a typical T3SS of the Hrp2 family (Fig. 2). The T3E

TABLE 2 PCR primers used to amplify T3E genes studied[a]

T3E gene	Forward primer	Reverse primer	PCR fragment size (bp)
*avrBs2**	ACCGCGCTGGCCACACCG	TCACTCGCCCGGCTCGATC	2,118
	TGCCGGTGTTGATGCACGA	TCGGTCAGCAGGCTTTC	850
*xopF1**	TGAAACTCACCAGCAATATCG	CTAGCGAAGCGCCTCGCTC	1,996
	AGGCCATCGACCCCAAGATCC	GTTCTTGGCCTTGAGCGCATTCC	779
*xopA**	ATGGACTCATCTATCGGAAACTT	GCCGGTGATGCTCGACAG	381
	TGCAGACGATGGGCATCG	CTGCATCAGCTGCATCACGATC	239
*hrpW**	ATGCAACGCATGCTCAGCGACAT	GTCTTCAGGTTCGCCAGCTTCAC	905
	AAGGTCGTCACCGCGC	GTCCTGCACGACCTTGTCT	399
*hpaA**	ATGATCCGGCGCATTTCGCCAG	TCATGCACGAATCTCCTGAGCGGC	816
	CGCTGGATGGCATGGACGACG	CGTCTGAGCGTCTGGTCGGCGGC	292
*xopR**	ATGCGCCTGAGTCAGTTGTTT	GTAGCCGTTGTCGATTGCCTCTT	1,230
	CGTGCGGCCCTGATCGC	GTAGCCCTGCATCATGCGTT	303
*xopN**	ATGAAGTCATCCGCATCCGTCGAT	CTCGATCGGTTCGGGCTACTCG	2,090
	GTCATGACCCAGGGCGC	GGTGATGGCGGTGTGCTG	864
*xopX**	ATGGAGATCAAGAAACAGCAAACCGC	GGCGACAGGCTTTGCACATATCTGG	1,865
	GTGGAAAACAACCTGGG	CCCCAGTTCATCGCC	827
*xopZ**	GCACTTGCGGATACTAATGCGG	GTCGACGAAGTCCTGCAATTGG	2,868
	TTCGGCCGCGGCTCGGC	GCACGGCATGGCGCGCTCC	1,012
*xopQ**	GTGCCCGCAGGCGCTCATGCAA	CCTTGGCGTGAACAGCATGCC	1,224
	ACCCCGACGATGT	TTGTTGTAGGCGCG	484
*xopK**	GACGCCCTTGCTTCAGCGAAC	TTCGGTGGCCAGCAACGTGCC	2,454
	CTCGGCATCCAGGGC	GACAAAGCCCTTGTTCCA	357
*xopV**	ATGAAAGTCTCCGCAACCCTT	TCAGGTTGCGAAAGGTGAGG	1,023
	ACACGCCTGTTCGTCTC	GCGATGTTCCATTTGTA	236
*xopL**	ATGCGACGCGTCGATCAACCG	CTACTGATGGCCTGAGGGTTCCG	1,863
	CCACCGACCGTGGGCGCTTCATCATTA	ACATCTGCACTGCCTTGGCCAGC	1,324
*xopAI**	ATGACTTCGGTAAGCCAGCGCGAATC	TCGATCTGGCTTTGATAAATCCTCAGAC	950
	AGAGCAGACCACGCCCTCTACG	GAATATTCTTCGGGAAGCGAGTGC	507
*avrXccA1**	GTGGTTCGCTGCGATGGC	TCACCCAGCCAGCGGG	813
	GATGGGCGGCACCG	ATCGCCACGCACCTG	163
*avrXccA2**	GCCGATGGCTGCCGCCGGCGCTA	TTGGTGTTCCAGTTCCGATCCAGG	1,442
	ACGGCCCGTTCTTTCCGCAAAGCC	CAACGGGCGCTCCGGCGACG	371
*xopAH**	ATGAAGAACACGTCTGTCCCT	CTACTTCTGCGTGGGAGGC	1,002
	GTGGCTCGCTGGCCCGATC	GCTGTAGCAGTTTCACTTGCTG	346
*XopG**	ATCGCAGCGAGGATTACCCG	AGACCATTCTCGGCACGAATAG	553
	AATCGGAAGGTGAGGCTGCTG	GCCAGACTTACTTTTCGGATCAAG	263
*xopAF**	ATGACAAATGGTATAAGTCGTTATTCTTATAAC	CTATTTAACAAGATCTGTTACAAACCTCACG	657
	ATAAGCCAATCAAGGGCGTTGG	ATTTCCCACTGCGCTCTGCGA	205
*xopE2**	TAAGGGATTCGCACTGGGCCG	CCAAGGCATCCAGGCGCTCTG	393
	GACAAGCGCGATGCAATCG	TAGGCCTGCCGCGCAGCAT	254
*xopE3**	ATGGGTTGCACTATCTCAACGACAAAC	TTACTGGCTCTGCTCGCCCAGCTGAT	1,071
	CCGACATTGCCGTCAGCGATCACG	AGCGTTCTTGGGTGTGTTGAGCATTTG	381
avrBs3	CTCAAGATTGCAAAACGTGGCGGC	GTCAGGCCATGGGCCTGGCACA	1,412
	AGAGCATTGTTGCCCAGTTATCTC	GTTCGGTGACGCCCACTCT	333
xopH	ATGGAGCGGGAGATGGC	TTATGCATTGTGGTCGAGC	315
	ATTGTGGTATGGGCCTAGGC	TGCTTGGCGTACTCGTAGAAT	220
xopB	ATGAAGGCAGAGCTCACACGA	TCAGGCGCGGGTTGGTGCGAAGTA	1,835
	AGCATTTGGCCCAAGCGCTTT	CGCTTCGGTTGTCGTCATATTGG	574
xopAG	ATGCGAACAAAAACCTCACTGC	CTAGGCGTTTCTGGTCATGGAC	1,542
	GGCAGACAACTTCTTTCTT	GAGGCAGGCAAGGTTGG	293
xopI	ATGACCCGAGCTTGGAGTTC	GTCGCAAGCATCATGCTGGCCGTGGC	1,045
	TCTGCGCTGCCGAATGATCT	CTGGAATCGATGGATCGGCTCC	789
xopAD	GCGCTGGCCGAGCGGCTG	CAACGCCGGCGCGGCGCTGT	3,357
	CGAGGCGGACCTGGGCGAGATGAG	AGGCTCCACGATGTCCTTGAG	1,471
xopAM	ATGGAGCCAGATGGAGCTGCATCTGGAC	TTACTCCTGGCCCGACGTGATCTGGC	2,344
	ATGCGATTGCTCATCTCTGCATGCTTGGC	ATGCAACGATCCAAGTGCGGCAACAT	2,020
xopAL1	ATGCCAAAGATCCATCGTTCC	CTACCACCTAAACCCGTTGC	918
	ATAAAGGTTCGCTCTTC	GCAAGTACTTCACTTTC	292
xopAL2	ATGCCCGTCAATCGATCTGGC	TTAGATGCTTTTTAAGAAATCCTTTG	882
	AATATGCATGAGCGGCGGTCGTAG	GGAGGATAAGACATCAAGGGGATTG	363

Continued on following page

TABLE 2 (Continued)

T3E gene	Forward primer	Reverse primer	PCR fragment size (bp)
xopAK	ATGTGCGTTGCCAGGCCTCAATC	CATGCCCAGGTTGATGGCATT	1,422
	TCCAAGCCAGCCGGACAATG	GATTTCGAAGCTGCCCAGCTCGT	302
xopAE	ATGTTCAATATAAATCGCTTACTGC	CTAACGGATGTCCCATTCCC	1,941
	CATCGGCCTCATGCAGGGGCTCAGG	ATCGCCGCATCCTGCGCCGCTTGTCC	978
xopE1	ATGGGACTATGCATTTCAAAGC	TCATCTCGCCACCGTGACAG	1,203
	CGTTCGCGACCGTCAACGCCTG	CAGAAAGCCTGCCTTGCATTGGTTCG	894
xopD	ATGGAATATATACCAAGATATGAAGC	CTAGAACTTTTTCCACCACTTGC	1,638
	ATGTGGACAATCTGCCGTCGC	GCACATGATCGCCGCAGGAAT	480
xopC1	GAAATTCCAAAGGATGCGGCATT	GGAGGGTTGATCGCCGTAAGAG	2,262
	AGATCATAAGTCCCTCGTGGG	GACGCTGCTTTATGCTCAG	1,029
xopC2	ATGAGCCTGTCCGAAAGCGC	TCACACGGGCAGCGGCTTGC	648
	TTGCGCGAAGGGGCAAG	TCGTGAATCTCGCAGACA	216
xopP	TTGAATCGACCAAGCAGGCCCCA	GTGACCGGTCTTTTGGCATGTACG	1,936
	GTCCGCGGAATGCTCGTCGAAAT	TCTTGAGCGCATCGGCTTCGCGCCGT	934
xopAA	ATGCAGATCAAAACCGCAGGC	TTATTCCGACTGAGGCACTGG	2,067
	ATCGCCGCCTTCCTGGGCAACCTGT	GCCATGAGGCCCTTGGACAGGACG	1,346
avrBs1	ATGTCCGACATGAAAGTTAATT	TTACGCTTCTCCTGCATTTG	1,338
	AAATCCACGCTGCATGACA	GGGCCAAGCCATGATAGTT	608
xopF2	TGAAGCTCCAACGCCAGAACAG	AGCGCCTGCTTCTGACGCAAGG	1,975
	GCGGCCGAGCAGGA	CGCGGCCTTCGCC	286
xopJ1	GGTCTATGCGTTTCAAAGCCGAGC	ATAGCTGTCTCCAGTGCTGATCGA	1,100
	GTACAACCGTCGTTACCCTGG	GTTGTCATGCCACTGATCGAACA	394
xopJ2	TATGCGTTCACTTGGCTTTGG	ATTTTCCTCAATCGAGATGCCTC	1,020
	AGTTCCGAAGCCATAACAAA	CGCACCCAGTTGGATGA	369
xopJ3	ATGTGCGACTCCATAAGAGTGC	TCAGGATTCTAAGGCGTGACG	1,122
	GCTGGCATAAAACAGGATTGCC	CCTTTAAGCAAATAGACGCCAA	412
xopJ4	ATGAAAAACATATTTAGGTCACTTG	TTAGCTACGACTCAACGCATG	1,080
	TCATCGATTCCCTTGAGGC	CGGAGTCAAACGGACCATA	411
xopJ5	GTGGTGGCGGCCCAGAATC	TTAGCTCCAGTACTCGGCGTC	708
	ATGGCATCGCGGTGGACG	CGACGTCGTGTGCTGGAA	356
xopO	ATGATCAACACTTCCGTCAAG	TCACCTGTTTATCCGACGAC	636
	CTCGAGCATTCACGGCGTACGG	CCGCAGGAACAACGTGGACG	235
xopT	ATGGCACCAGCTCCGTGGAG	TCAATTCCCATGGCGGTCCAAC	882
	ATGCAGTTTCTGGATATACCAGAC	GTCACCATCTGACGCTTGGACA	524
xopW	GTGATGAAACCGAGCCACATCG	TCAACTGCCGCTACTGGAGGC	615
	ATGATTGAGGAGTACGGGCGCAAGAC	AGCTTCCAGCTCACGAAGACGACTT	279
xopY	ATGCGCCCTGTCCAGCCC	TTAGCTCAGCCGCCGGAAGA	846
	ATGAAGCGCTATGGGGATCAGACG	AGTTCTCCGACGGTGCTGCAGGT	358
xopAB	GTGCCACGGCATGTGCGATG	AGTCCTTGGCGTGTTTGCCG	574
	TTGATTCCGCAGGTGACC	GCTCAGGCTGCTCGGGG	239
xopAJ	GTGGGTGTGCGAGTCGC	TCAAATTAGCTCGCTATGAGC	1,353
	ATCTCTTGAGCGCGATGGC	GCCTAGGATCTTCACCTTCA	455
xopU	ATGGATGCCCTGCTGCGTG	GCGCGCCGACGCTGCC	2,951
	ATGCTCTGCTCGATCCCCTTC	CAAGGGCCGGCATGAAGGCG	927
xopAC	ATGGATAAAAATCTTAATTTGTGG	CTACTGGTGAACCTGGTTC	1,611
	TGAACTGAGAACCCTTCCAG	TCCGGAAAACTTCGCCATT	646

[a] For each T3E gene, two sets of primers allowing amplification of full-length and partial T3E DNA sequences were designed. For genes with an asterisk, primers were designed directly on the genome of *X. arboricola* pv. pruni strain CFBP5530. For the remaining T3E genes, primers were designed on conserved regions of T3E genes in *Xanthomonas* sequenced genomes. For *xopJ2* and *xopJ4*, primers were designed on *X. axonopodis* pv. vesicatoria strains whose genome have not been sequenced: strain 75-3 and strain 91-118.

gene distribution analysis revealed that T3E repertoires vary in size between *X. arboricola* pathovars (Fig. 2). Strains of *X. arboricola* pv. pruni, *X. arboricola* pv. corylina, and *X. arboricola* pv. juglandis exhibited the largest T3E repertoires (from 18 to 22 out of the 53 T3E genes studied depending on the pathovar), whereas strains of *X. arboricola* pv. celebensis, *X. arboricola* pv. poinsettiicola, and *X. arboricola* pv. fragariae exhibited the smallest (6 out of the 53 T3E genes studied).

The composition of T3E repertoires differs between *X. arboricola* pathovars. Our study also revealed many differences between *X. arboricola* pathovars in the composition of their T3E repertoires. In common with all previously sequenced *Xanthomonas* genomes, *X. arboricola* pathovars (pruni, corylina, juglandis, poinsettiicola, celebensis, and fragariae) possessed orthologues of *avrBs2*, *xopF1*, *xopA*, *hrpW*, *hpaA*, and *xopR* (Fig. 2). These genes will be referred to as the ubiquitous set of T3E genes for strains of *X. arboricola* that might have been acquired before the radiation of this species into the current pathovars. In addition, strains from *X. arboricola* pv. pruni, *X. arboricola* pv. corylina, and *X. arboricola* pv. juglandis possessed orthologues of *xopN*, *xopX*, *xopZ*, *xopQ*,

TABLE 3 Features of sequenced T3E genes from *X. arboricola* pathovars and positions and identities of discriminative amino acids between different alleles

T3E gene	*X. arboricola* pathovar(s)	CFBP strain no.	Sequence length (bp)	Positions of substitutions/mutations[a]	Substitutions/ mutations[b]
xopA	pruni, corylina, juglandis	3894, 5530, 5575, 5722, 5724, 7100, 1159, 1847, 2565, 5956, 7381, 7384, 2528, 2568, 2632, 12581, 12765, 12785	327	16, 98	E, S
	celebensis, poinsettiicola, fragariae	3523, 7150, 7152, 7278, 3548, 6762, 6763			Q, G
xopQ	pruni	3894, 5530, 5575, 5722, 5724	846	65, 123, 203	C, D, T
	corylina, juglandis	1159, 1847, 5956, 7381,7384, 2528, 2568, 12581, 12765, 12785			G, A, A
xopAI	pruni	3894, 5530, 5575	837	6, 8, 88, 94, 198, 224, 240	I, L, R, *, H, D, G
	corylina, juglandis	1159, 5956, 7384, 2568, 12765, 12785			M, V, H, R,Q, G, A
xopV	corylina	1159, 5956	852	31, 150, 153, 228, 258, 268	D, Y, N, *, V, K
		7381			D, Y, N, C, G, R
		7384			D, H, N, C, G, K
		1847			N, Y, K, C, G, K
	pruni, juglandis	3894, 5530, 5575, 5722, 5724, 2528, 2568, 12581, 12765, 12785			D, Y, N, C, G, K
xopG	pruni, corylina	3894, 5530, 5575, 1159, 1847, 5956	438		
xopE3	pruni	3894, 5530, 5575, 5722, 5724, 7100	930		
xopH	corylina	1847, 1848, 2565, 7381, 7384, 7385	309		
xopB	juglandis	12585, 12763, 12765, 12772, 12784, 12785	1,392		

[a] Positions of mutations are given with respect to the protein alignments generated from nucleotide sequences.
[b] Mutations correspond to nonsynonymous amino acid changes. *, nonsense mutation.

xopK, and *xopV*, which were also shown to be conserved in a subset of the previously sequenced *Xanthomonas* genomes (http://www.xanthomonas.org/t3e.html), and *xopL*, *xopAI*, *avrXccA1*, *avrXccA2*, and *xopAH*, which were shown to be more heterogeneously distributed among sequenced *Xanthomonas* genomes (http://www.xanthomonas.org/t3e.html). Some T3E genes were present in only two *X. arboricola* pathovars: this was the case of *xopG*, *xopAF*, and *xopE2*, which were detected in *X. arboricola* pv. pruni and *X. arboricola* pv. corylina strains (Fig. 2). This second class of genes will be referred to as variable T3E genes for strains of *X. arboricola*. Furthermore, some T3E genes were detected in only one of the *X. arboricola* pathovars studied and will be referred to as specific T3E genes. For example, *xopE3* was detected only in strains of *X. arboricola* pv. pruni and *xopB* in VOC strains of *X. arboricola* pv. juglandis, and *avrBs3* and *xopH* were present only in strains of *X. arboricola* pv. corylina. A total of 29 out of the 53 T3E genes studied were not detected in any *X. arboricola* strains with the two specific PCR primer sets (Fig. 2).

A few variations in T3E repertoires occurred between strains of the same pathovar. The T3E gene distribution analysis revealed that T3E repertoires were conserved in most pathovars. Indeed, we observed identical T3E assortments in strains belonging to pathovars pruni, celebensis, poinsettiicola, and fragariae, whereas we observed some variations in T3E repertoires in pathovars corylina and juglandis (Fig. 2). Interestingly, the variation observed within pathovar corylina can be linked to the host of isolation. Indeed, among *X. arboricola* pv. corylina strains, T3E repertoires were almost identical, but strains isolated from *C. avellana* carried one more T3E gene (*xopH*) than strain CFBP1159, isolated from *C. maxima* (Fig. 2). The observed variation in T3E repertoires among *X. arboricola* pv. juglandis strains can be linked to the genetic diversity reported within this pathovar. The two genetic lineages (WB/VOC) defined within pathovar juglandis possess similar but not identical T3E repertoires, since in addition to a core set of 16 T3E genes, each genetic lineage harbors an additional T3E gene: *xopB* in VOC strains and *xopAH* in WB strains (Fig. 2).

Limited allelic variations were observed in T3E genes of *X. arboricola* pathovars. To gain insight into sequence variation among orthologues of T3E genes in different *X. arboricola* pathovars, we chose to sequence eight T3E genes from a subcollection of strains representative of the biodiversity of the different pathovars. In total, a subset of 88 sequences of ubiquitous (*xopA*), variable (*xopV*, *xopQ*, *xopAI*, and *xopG*), and specific (*xopE3*, *xopH*, and *xopB*) T3E genes was obtained. Overall, very limited allelic variability was observed for the eight T3E genes, as revealed by the corresponding neighbor-joining trees built from nucleotide alignments (Fig. 3).

The neighbor-joining tree corresponding to the alignment of 327 bp of the ubiquitous T3E gene *xopA* clearly distinguished two groups of alleles. The first group included representatives of stone

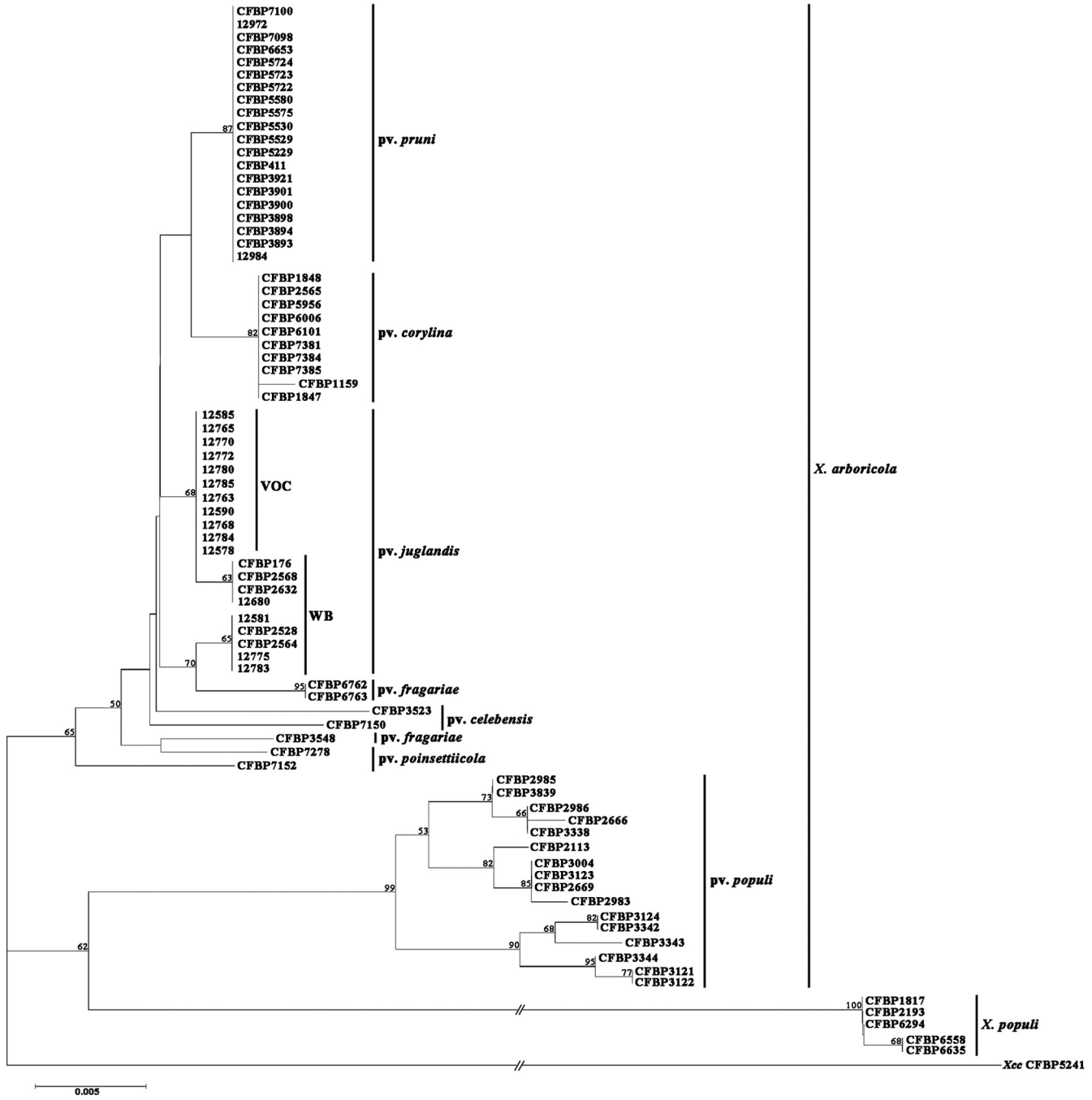

FIG 1 Phylogeny of the species of *X. arboricola* based on *rpoD* gene sequences. WB and VOC refer to strains of *X. arboricola* pv. juglandis causing walnut blight and vertical oozing canker, respectively. The phylogenetic tree was constructed using the neighbor-joining method. The confidence of nodes was tested with 1,000 bootstrap replicates. Bootstrap values under 50 are not shown. The scale represents the number of substitutions per site. The tree is rooted with the *rpoD* gene sequence of strain CFBP5241 of *X. campestris* pv. campestris. *X. populi* strains were also included in the phylogenetic analysis.

fruit and nut pathogens (pathovars pruni, corylina, and juglandis) whose nucleotide sequences were completely identical (Fig. 3) and were distinguished from the second group of alleles by a 6-bp deletion (GGCGTG) at position 277 of the *xopA*-coding region and two encoded amino acid substitutions (Table 3). However, alleles in the second group, which included strains of pathovars celebensis, poinsettiicola, and fragariae, were more variable at the nucleotide level (Fig. 3) even though they were found to encode identical proteins.

For T3E genes showing a variable distribution in *X. arboricola*, different cases were observed. Concerning *xopQ* and *xopAI*, the neighbor-joining trees obtained after the respective alignment of 846 bp and 837 bp enabled the definition of two groups of alleles (Fig. 3). Indeed, alleles identified in *X. arboricola* pv. pruni strains were distinguished from those identified in strains of *X. arboricola* pv. corylina and *X. arboricola* pv. juglandis on the basis of seven and nine nucleotide substitutions, respectively, some of which caused amino acid substitutions (three and six, respectively) (Ta-

TABLE 4 PCR primers used to amplify structural and regulatory components of the T3SS belonging to the Hrp2 family[a]

Gene	Forward primer	Reverse primer	PCR fragment size (bp)
hrcR	GCTGGTGGTCATCATGCTGG	GTGTTTGAGGAGGAATTGC	292
hrcN	ATGTCAACGTGATCGTGC	CTGGCTCATCACCCGGCTC	524
hrcT	GTCGTTCTACGCGCTGG	GTTGGCGGCATCGTGCAA	376
hrcC	ACCGAAGTGCAGGTGTTTC	ATCTCGATGATGGTGGCATCGAT	575
hrcV	GCGCCATGAAATTCGTCAAGG	GCCAGCAGCAGGAACAGC	367
hrcU	GGCGTGGTGCTGTGG	GGTTGACCACCATCACCTTG	340
hrcS	TTGCCGGTGGTCGG	AACGCCGCCTGCATCA	191
hrcJ	CTCGGCGAGATGTTCAAG	GCCACCAATACAGCGC	436
hrpB1	CTGATCACGGTCGG	TCGGCATCGGCGTC	287
hrpD5	GGCGCACGATTAGATTTG	TGCTGCGGCAGGGA	887
hrpF	ACGCTGGACACCATC	TTCTTGTAGCCGGTGAT	188

[a] For each gene, primers were designed on conserved regions in *Xanthomonas* sequenced genomes.

ble 3). Concerning *xopV*, the generated sequences were highly conserved, and only *X. arboricola* pv. corylina alleles differed from *X. arboricola* pv. pruni and *X. arboricola* pv. juglandis alleles (Fig. 3; Table 3). Sequences of *xopG* were found to encode identical proteins in *X. arboricola* pv. pruni and *X. arboricola* pv. corylina strains, since the single base nucleotide substitution resulted in a synonymous mutation at the protein level (Fig. 3; Table 3). In several cases, the sequences revealed the presence of premature

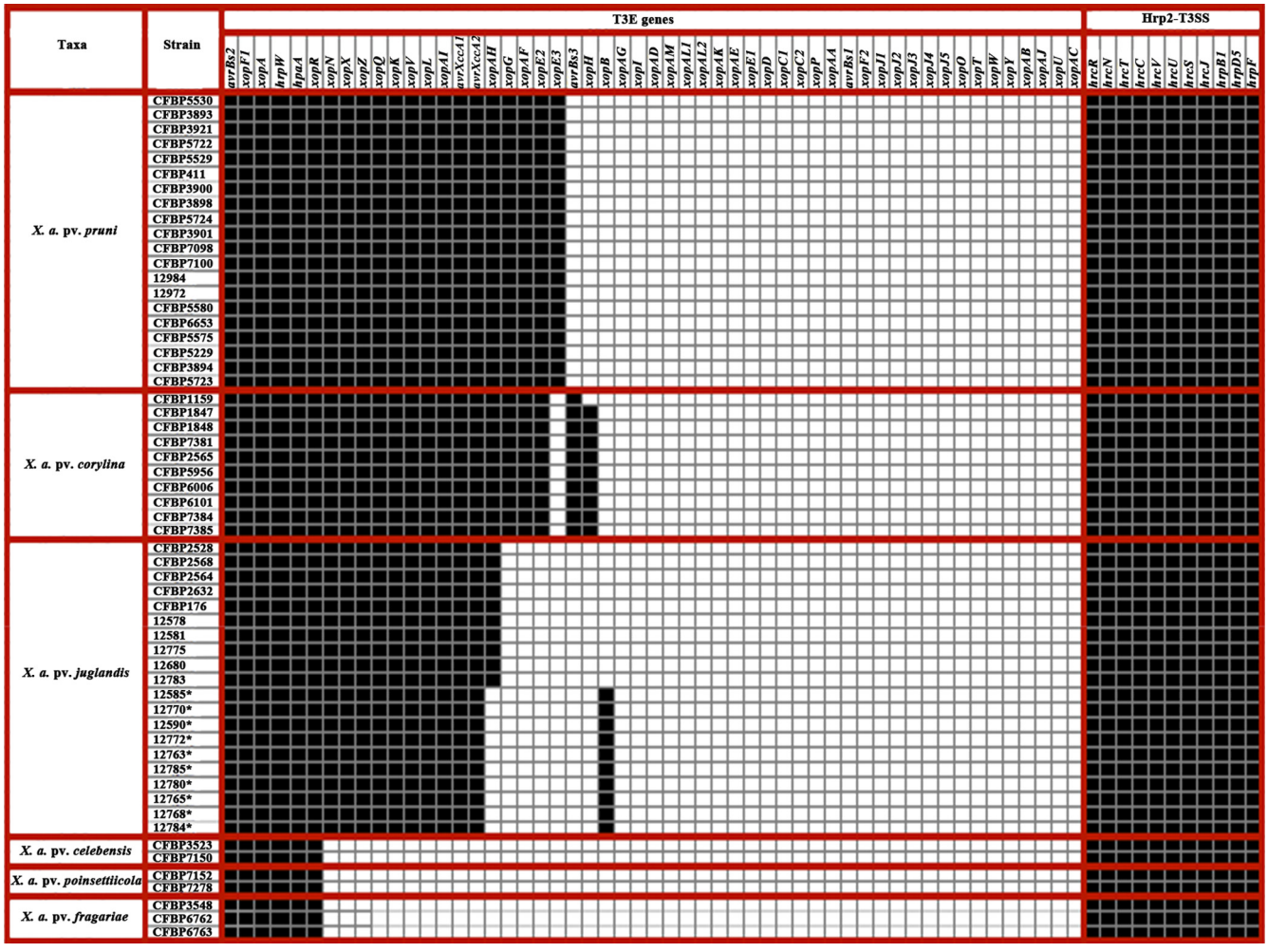

FIG 2 Distribution of 53 T3E genes among 57 strains belonging to six pathovars of *X. arboricola*. The nomenclature and classification scheme used for T3E genes in xanthomonads is available at the *Xanthomonas* resource website (http://www.xanthomonas.org/t3e.html). The distribution of 11 genes (3 are *hrp*, and 8 are *hrp* conserved [*hrc*]) coding for the structural and regulatory components of the T3SS of the Hrp2 family is also presented. The presence or absence of an orthologue of each selected gene was determined by PCR. Black squares represent the presence of the corresponding gene; white squares represent the absence of the gene. *X. arboricola* pv. juglandis strains that belong to the VOC cluster on the basis of f-AFLP analysis (24) are shown with an asterisk.

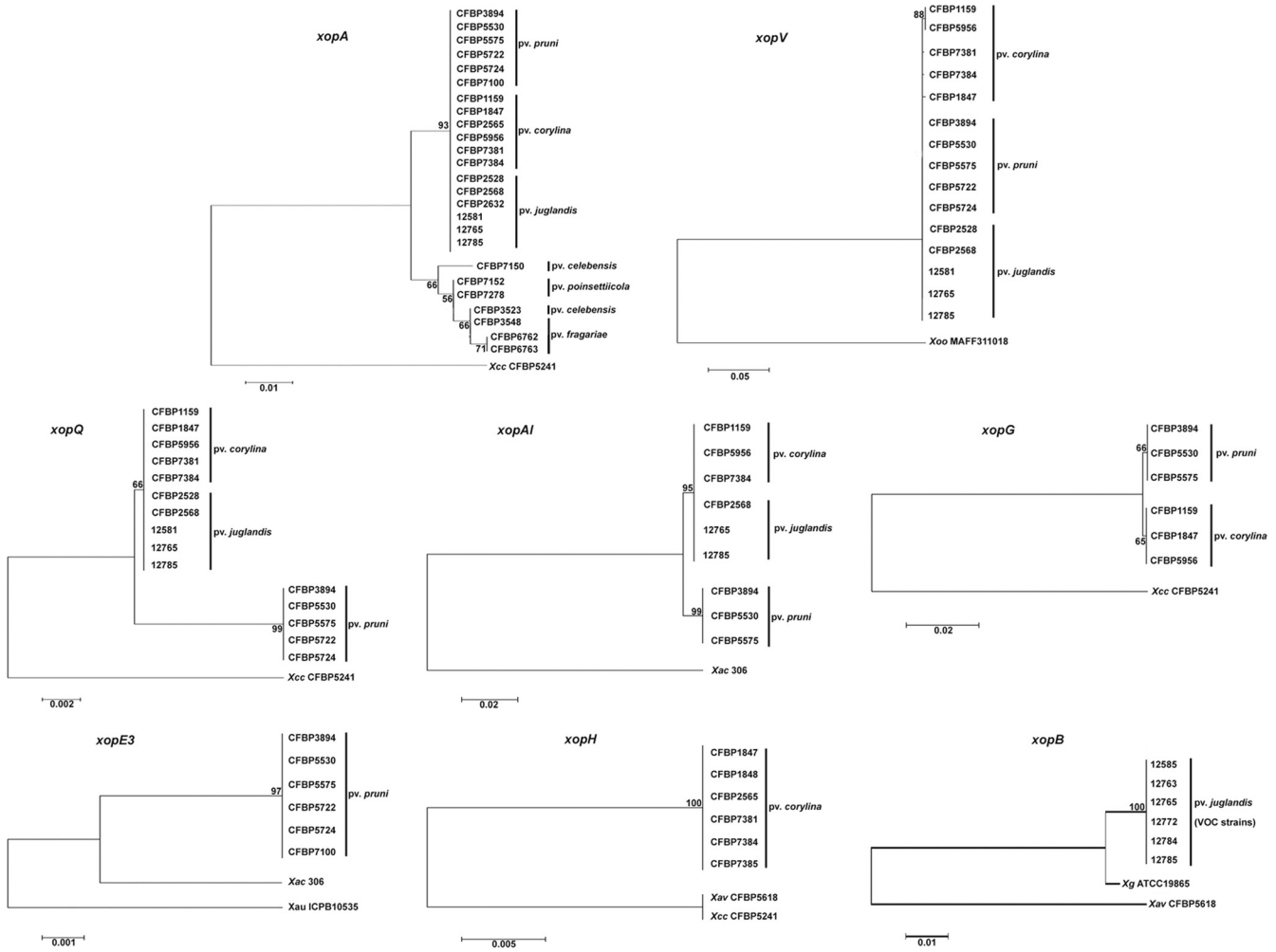

FIG 3 Phylogenetic analyses of eight T3E genes from *X. arboricola* pathovars. Trees were inferred using the neighbor-joining method from aligned nucleotide sequences of T3E genes from *X. arboricola* pathovars. Bootstrapping was performed with 1,000 replicates. The scale represents the number of substitutions per site. The trees are rooted with T3E gene sequences from *Xanthomonas* sequenced genomes. Abbreviations for strains used as outgroups are as follows: *Xcc* CFBP5241, *Xanthomonas campestris* pv. campestris; *Xoo* MAFF311018, *Xanthomonas oryzae* pv. oryzae; *Xac* 306, *Xanthomonas axonopodis* pv. citri; *Xav* CFBP5618, *Xanthomonas axonopodis* pv. vesicatoria; *Xau* ICPB10535, *Xanthomonas fuscans* subsp. *aurantifolii*; *Xg* ATCC 19865, *Xanthomonas gardneri*.

stop codons. This was the case for *xopAI* in all *X. arboricola* pv. pruni strains tested (CFBP3894, CFBP5530, and CFBP5575) and for *xopV* in two *X. arboricola* pv. corylina strains (CFBP1159 and CFBP5956) (Table 3). For specific T3E genes (*xopE3*, *xopH*, and *xopB*), the six nucleotide sequences obtained, respectively, from pathovars pruni, corylina, and VOC strains of pathovar juglandis were completely identical (Fig. 3).

DISCUSSION

The purpose of the present study was to investigate the variability of T3E repertoires in the species *X. arboricola* and their potential role in structuring its populations according to the host range. To our knowledge, this is the most complete T3E gene distribution analysis in *Xanthomonas*, since we selected all 53 known T3E genes available at the *Xanthomonas* resource website (http://www.xanthomonas.org/t3e.html). The most important findings of this study concerned the characterization of T3E repertoires in a species for which no published genome sequences are available. Our results confirm the emerging view that, in plant-pathogenic bacteria, T3E repertoires comprise a core set of common T3E genes carried by most strains and a variable set of T3E genes that can be correlated with a pathovar (19, 21, 23, 54, 57). Interestingly, this study clearly revealed the existence of a presence/absence polymorphism of T3E genes between the three important stone fruit and nut pathogens (pathovars pruni, corylina, and juglandis) with differing host specificities, thus confirming that these *X. arboricola* pathovars are different genetic entities (46, 53, 61, 72, 75). In addition, we uncovered numerous pathovar-specific features (*xopE3* in *X. arboricola* pv. pruni, *xopB* in VOC strains of *X. arboricola* pv. juglandis, and *avrBs3* and *xopH* in *X. arboricola* pv. corylina). These genes are thus candidates for functional analyses to determine whether they play a significant role in virulence and in the host range of these pathovars. We now plan to point our work toward functional studies that will aim at showing gain or loss of function. For instance, focusing on pathovars pruni and corylina may be an excellent approach since these pathovars are phylogenetically closely related as shown by our *rpoD* sequence analysis. Furthermore, these two pathovars harbor similar but not identical

T3E repertoires, since in addition to a core set of 20 T3E genes, each pathovar harbors few additional T3E genes: *xopE3* (present in pathovar pruni and absent in pathovar corylina) and *avrBs3 and xopH* (present in pathovar corylina and absent in pathovar pruni). It would be interesting to observe whether the host range is enlarged or narrowed if the variable T3E genes in pathovar corylina are transferred into pathovar pruni or if they are deleted. The same kind of functional studies might be performed with the variable TE3 gene *xopE3* in pathovar pruni.

Among *X. arboricola* pathovars, it should be noted that *X. arboricola* pv. corylina is the only pathovar that contains homologues of the *avrBs3* gene. The *avrBs3* gene is a member of the transcription activator-like (TAL) effector family, also called the AvrBs3/PthA family, whose members are present in most *Xanthomonas* genomes and in some *Ralstonia solanacearum* genomes (http://www.xanthomonas.org/t3e.html). Individual strains of *Xanthomonas* are known to contain multiple copies of the AvrBs3/PthA genes, up to 28 paralogs in some *X. oryzae* pv. oryzicola strains (http://www.xanthomonas.org/t3e.html). Our PCR approach has a limit since it cannot reveal the number of copies of these genes. In addition, evidence of a direct relationship between the presence of AvrBs3/PthA members in *X. arboricola* pv. corylina and the ability of this pathogen to provoke cankers was not investigated in this study but is well documented for *X. axonopodis* pv. citri. Indeed, PthA is known to be an important T3E in *X. axonopodis* pv. citri which plays an essential role in citrus canker, since a deletion of *pthA* abolishes the ability of this bacterium to cause cankers (3, 67). To get insight into the evolution of the AvrBs3/PthA family gene and to further investigate the role of this family gene in pathogenicity of pathovar corylina, it would now be interesting to determine by Southern hybridization the number of copies of AvrBs3/PthA genes in *X. arboricola* pv. corylina strains and to compare AvrBs3/PthA gene homologues from *X. arboricola* pv. corylina strains to all available family members in the genus *Xanthomonas*.

The major conclusions reached in earlier population structure and phylogenetic studies concerning some *X. arboricola* pathovars (11, 24, 36, 46, 53, 59, 60, 61, 72, 75, 76) are reinforced by the results of the present study. Our phylogenetic analysis of the species *X. arboricola* reveals two major groups of strains, with *X. arboricola* pv. populi strains found to be separated from the coherent main *X. arboricola* cluster formed by all other strains (from pathovars pruni, corylina, juglandis, poinsettiicola, celebensis, and fragariae). To further elucidate the taxonomic status of *X. arboricola* pv. populi strains, more substantive data on its population genetics are now needed. For this purpose, we are now carrying out an MLSA approach on the species *X. arboricola* based on the sequencing of seven housekeeping genes. Our findings revealed that strains of *X. arboricola* pv. pruni have indistinguishable T3E repertoires, even though they originated in 10 countries and were isolated from seven host species over a 60-year time span (Table 1). Consequently, no relationship with the geographical location or host of isolation was demonstrated for this pathovar. One possible explanation for the observed homogeneity of the T3E repertoires among pathovar pruni is that *X. arboricola* pv. pruni strains are very close phylogenetically as shown by our *rpoD* sequence analysis. Our data reinforce the hypothesis that *X. arboricola* pv. pruni strains belong to a single epidemic population with very low genetic diversity which was able to maintain itself for years and to disperse all over the world (11). In this study, we revealed many common features and one difference in T3E gene content between *X. arboricola* pv. corylina strains, since those isolated from *C. avellana* harbored *xopH*, which was absent in strain CFBP1159, isolated from *C. maxima*. The distinctive nature of strain CFBP1159 is supported by our *rpoD* sequence analysis and was previously reported on the basis of a rep-PCR analysis and pathogenicity tests (60). Typing of more strains isolated from *C. maxima* is now necessary to confirm this result. In addition, the *X. arboricola* pv. juglandis strains we tested displayed two T3E repertoire patterns that are linked with different genetic lineages (WB and VOC) (24), since we showed that *xopAH* and *xopB* enabled these two genetic lineages to be distinguished. Such findings could mean that adaptation to particular environments encountered by some genetic lineages within certain pathovars (juglandis in this case) can be putatively linked to different T3E repertoires even though the function of these two differential genes in xanthomonads is still unknown.

Vertical oozing canker (VOC) is an emerging disease which threatens walnut cultivation in France, especially in nurseries. The causal agent of the disease was identified as a distinct pathogenic genetic lineage within *X. arboricola* pv. juglandis by fluorescent AFLP (f-AFLP) analysis (24). Our *rpoD*-based tree grouped VOC strains within a cluster that was clearly distinct from WB strains, supporting the hypothesis based on earlier phylogenetic analysis that VOC strains differ from WB strains (24). Interestingly, our study revealed that VOC strains harbor *xopB*, which is absent from WB strains. This T3E shows sequence similarity to the C terminus of the avirulence protein AvrPphD of the bean pathogen *P. syringae* pv. phaseolicola (42). This gene is not broadly distributed in *Xanthomonas*, since it has been found in only a few *X. axonopodis* pathovars, such as *X. axonopodis* pv. begoniae, *X. axonopodis* pv. vasculorum, and *X. axonopodis* pv. vesicatoria (23). The G+C content of *xopB* in VOC strains (57.1%) was lower than the average value for *Xanthomonas* (~65%), suggesting that it could have been acquired through horizontal gene transfer. Our results will serve as a starting point for molecular and functional studies of this recently emerging disease. We hypothesize that the acquisition of a novel T3E gene (*xopB* in this case) may confer new pathogenic abilities to VOC strains, but clear evidence supporting this hypothesis is currently lacking. It would be interesting to delete *xopB* from VOC strains to determine whether *xopB* plays a role in the ability of these strains to cause VOC in walnut. However, a negative result may simply mean that other T3E genes in VOC strains have redundant functions, since a mutation in a single T3E gene could have no detectable effect on pathogenicity, as was shown for several plant-pathogenic bacteria (31, 58).

In plant-pathogenic bacteria, T3E repertoires are known to be highly dynamic components of the genome. The evolution of T3E repertoires is due to different mechanisms, including single-nucleotide polymorphisms (SNPs), acquisition and loss of T3E genes, transposon insertions, and terminal reassortment (7, 37, 44, 48, 62, 64). To assess the evolutionary mechanisms that might shape the evolution of T3E repertoires in the species *X. arboricola*, we examined the sequences of ubiquitous, variable, and specific T3E genes. Overall, we found extremely reduced allelic variability among *X. arboricola* pathovars for the eight sequenced T3E genes. It is worth noting that the ubiquitous *xopA*-based neighbor-joining tree is consistent with the phylogeny of the species *X. arboricola* established on the basis of our *rpoD* sequence analysis and other phylogenetic studies (46, 53, 72, 75). This result suggests

that *xopA* was likely acquired before the divergence of contemporary pathovars within *X. arboricola*. Concerning variable T3E genes, we detected several nucleotide mutations, some of which resulted in amino acid substitutions. In addition, some polymorphic sites exhibited a frameshift mutation leading to a premature stop codon. The most striking example was *xopAI*, with one allele carried by stone fruit pathogen strains (*X. arboricola* pv. pruni) and differing by six amino acid substitutions and one nonsense mutation from the allele carried by nut pathogen strains (*X. arboricola* pv. corylina and *X. arboricola* pv. juglandis). Whether these differences are significant from an evolutionary or functional perspective was not investigated in this study but remains to be determined. The presence of *xopAI* was previously found in only three *Xanthomonas* sequenced genomes, associated with citrus, and in *X. vesicatoria* strain ATCC 35937 (39, 50). Our study provides the first evidence of its presence in nonsequenced *Xanthomonas* genomes. Interestingly, the N-terminal region of XopAI shows high similarity with the N terminus of XopE2 of *X. axonopodis* pv. *vesicatoria* strain 85-10, a member of the HopX/AvrPphE effector family from *P. syringae* (35, 39, 70). This gene family provides a nice illustration of the importance of pathoadaptive changes in host-pathogen interactions, since all the alleles carrying nonsynonymous mutations in *P. syringae* pv. phaseolicola resulted in a loss of avirulence, thereby shifting the interaction from incompatible to compatible (41, 65).

In our study, many T3E genes were detected in only one of the *X. arboricola* pathovars analyzed. The absence of these genes in one or more genomes could be the result either of its presence in the ancestor followed by loss or of its absence in the ancestor followed by acquisition by horizontal gene transfer. The second hypothesis is supported by the fact that these genes have identical DNA sequences within the same pathovar as shown by our sequence analysis and G+C content significantly lower than the average value in *Xanthomonas* genomes (~65%), suggesting that they were most likely acquired by horizontal gene transfer. This was the case for *xopE3* (58.6%) in *X. arboricola* pv. pruni strains, *xopH* (43.2%) in *X. arboricola* pv. corylina strains, and *xopB* (57.1%) in VOC strains of *X. arboricola* pv. juglandis. This hypothesis is corroborated by the fact that in other *Xanthomonas* strains, some of these T3E genes are associated with mobile genetic elements, such as insertion sequences. For instance, the *xopE3* gene was previously shown to be interrupted by IS*Xca2* in two strains of *X. axonopodis* pv. alfalfae (23). Since many T3E genes were also shown to be disrupted by insertion sequences (ISs) in *X. axonopodis* strains (23), in *P. syringae* (37), or in *R. solanacearum* (32), we can hypothesize that inactivation of T3E genes by ISs may play a significant role in the pathological adaptation of these phytopathogenic bacteria to their hosts. Further sequencing of more T3E gene product polymorphisms from *X. arboricola* pathovars is now necessary to provide resources for determining the driving forces shaping the evolution of T3E repertoires.

In this article, we have described the genetic basis of host specificity of the complex species *X. arboricola*, with special emphasis on T3E repertoires. Our study clearly revealed a close correspondence between the composition of T3E repertoires and *X. arboricola* pathovars and thus confirms our previous findings obtained with a collection of *X. axonopodis* and *X. oryzae* strains (23, 25). The characterization of T3E repertoires of *X. arboricola* pathovars also provides clues for functional studies of virulence and host specificity of these pathogens. However, because T3E repertoires do not explain all differential host specificities, it is important to elucidate the repertoires of other pathogenicity determinants possibly involved in ecological specificities and population structuring of *X. arboricola* pathovars. Among them, particular attention should be paid to genes involved in adhesion and sensing, biofilm formation and quorum sensing, type IV secretion system (T4SS), flagellum synthesis, motility, and lipopolysacharide synthesis (12, 38). Finally, further studies, taking into account the overall population structure of the species *X. arboricola* by sequencing seven housekeeping genes, are now being undertaken to propose a tentative scenario for evolutionary history and pathovar diversification within this complex bacterial species.

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