

Phylogenetic insights derived from six *Xanthomonas* draft genome sequences associated with bacterial spot disease of tomato and pepper in Turkey

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Abstract

Xanthomonas spp. are increasingly recognized as a global threat to agriculture, impacting a broad range of economically important crops. We report the whole-genome sequences of six *Xanthomonas* strains isolated from tomato and pepper plants in Turkey that were experiencing symptoms of bacterial spot disease. Phylogenomic analysis with representative *Xanthomonas* genomes from each species revealed that three of these strains belonged to *Xanthomonas perforans*, two to *Xanthomonas euvesicatoria* and one to *Xanthomonas campestris*. We then analysed the phylogenomic relatedness of these strains with other strains from these respective species and characterized their type III secreted effector content. These genomic data represent a valuable resource for understanding the genetic diversity and local epidemiology of bacterial spot disease in Turkey.

DATA SUMMARY

Whole-genome assemblies and raw read data have been deposited in GenBank under BioProject number [PRJNA1353131](#). Genome accessions for all genomes are listed in Table 1.

INTRODUCTION

Xanthomonas is a complex genus of phytopathogenic bacteria comprising more than 40 species that infect a wide range of plant hosts. Members of this genus exhibit strong host specificity and high genetic diversity both within and between species, making *Xanthomonas* an excellent model system for studying plant–pathogen interactions and understanding the evolution of emerging plant pathogens [1]. Bacterial spot of tomato and pepper is an economically important disease that poses a significant threat to global tomato and pepper production. This disease is primarily caused by four *Xanthomonas* taxa: *Xanthomonas euvesicatoria* pv. *euvesicatoria* (syn. *X. euvesicatoria*), *Xanthomonas hortorum* pv. *gardneri*, *Xanthomonas euvesicatoria* pv. *perforans* (syn. *X. perforans*) and *Xanthomonas vesicatoria*. These species have undergone considerable evolutionary diversification and structural evolution over time, with their global distribution and prevalence varying substantially across tomato and pepper growing regions [2]. Under optimal environmental conditions, these pathogens can cause severe yield losses in transplants or field production areas [3]. However, management of bacterial spot disease has been a major challenge due to the rapid evolution of these species, the widespread resistance to bactericides, and the lack of resistant cultivars.

Turkey is a leading global producer of tomato and pepper, ranking third in tomato production and fourth in pepper production worldwide (FAO, 2024). The Adana and Mersin provinces are both located in the Mediterranean region and are among the top five tomato and pepper producing provinces in Turkey, with extensive cultivation under both field and greenhouse conditions (Ministry of Agriculture and Forestry, 2024). Kayseri province, which is adjacent to Adana, is also a major producer of tomatoes, producing 24,921 tonnes of table tomatoes and 55,535 tonnes of processing tomatoes per year [4, 5]. Bacterial spot of pepper and

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Abbreviations: ANI, average nucleotide identity; ETI, effector triggered immunity; KB, King's B; NCBI, National Center for Biotechnology Information; T3SEs, type III secreted effectors.

A supplementary table is available in the online Supplementary Material.

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Table 1. Metadata and genome statistics for the six new *Xanthomonas* strains from Turkey that were isolated from tomato and pepper plants experiencing bacterial spot symptoms

Species	Strain	Host	Source	Location	Year	Genome length (Mbp)	No. of contigs	N50 (bps)	Genome accession
<i>X. perforans</i>	YA993	Pepper	Nursery	Adana	2020	5.04	30	465,574	JBTKYF000000000
<i>X. perforans</i>	YA1076	Pepper	Nursery	Adana	2021	5.05	31	392,180	JBTKYE000000000
<i>X. perforans</i>	YA1054	Tomato	Nursery	Adana	2021	5.05	34	389,719	JBTKYD000000000
<i>X. euvesicatoria</i>	YA1087	Pepper	Field	Adana	2021	5.05	65	222,486	JBTKYC000000000
<i>X. euvesicatoria</i>	YA1525	Pepper	Nursery	Mersin	2024	5.30	114	163,507	JBTKYB000000000
<i>X. campestris</i>	YA1028	Pepper	Field	Kayseri	2020	5.07	82	142,846	JBTKYA000000000

tomato was first reported in the Eastern and Western Mediterranean regions of Turkey in the early 2000s, with field and greenhouse outbreaks being regularly reported since then [6, 7]. This has resulted in significant productivity losses, particularly in the hot and humid regions of the Mediterranean, where disease prevalence can be as high as 100% in some years. A recent study by Subedi *et al.* reported the genome sequences of ten *Xanthomonas* strains associated with bacterial spot disease on pepper and tomato from the Southeastern Anatolia Region [8]. However, genomic information on bacterial spot pathogens from the Mediterranean region of Turkey remains limited. A more complete picture of the population genomics of *Xanthomonas* pathogens causing bacterial spot in Turkey requires that we sample a more diverse collection of strains across high-yield production regions with diverse climates.

In this study, we sequenced six additional strains isolated from bacterial spot outbreaks on tomato and pepper plants in the Mediterranean region of Turkey and evaluated their evolutionary context in the *Xanthomonas* genus using a phylogenomic approach. The six new strains that we sequenced were assigned three distinct *Xanthomonas* species: *X. euvesicatoria* (2), *X. perforans* (3) and *Xanthomonas campestris* (1). A more fine-scale phylogenomic analysis with other strains from these species provided additional evidence of local adaptation, ongoing host range expansion and strain-level diversity, which is likely driven by horizontal gene transfer. Furthermore, analysis of the type III secreted effector (T3SE) repertoires of these strains provided additional insight into their role in strain- and species-level diversification.

METHODS

Sample collection

The six bacterial strains used in this study were isolated from diseased tomato and pepper plants collected from nursery or field grown plants between 2020 and 2024 in three Turkish provinces: Adana (4), Mersin (1) and Kayseri (1) (Table 1). Bacterial isolation was performed following the protocol described by Dillon *et al.* [9]. Briefly, symptomatic plant leaves were surface sterilized with 70% ethanol and macerated in sterile 0.85% (w/v) NaCl saline for 20 min. A loopful of resulting suspension was then streaked on King's B (KB) agar plates and incubated at 28°C for 48 h. Single colonies were then cultured in liquid KB medium for 48 h and stored in 8% DMSO at –80°C. For genomic DNA extraction, a single colony was grown in 5 ml of liquid nutrient yeast glycerol medium for 48 h at 28°C. DNA was then extracted from a 1 ml culture using the PureLink Genomic DNA Extraction Kit (Invitrogen), and DNA concentration and quality were verified using the BioTek Synergy LX with the Take3 plate (Agilent Technologies).

Whole-genome sequencing and assembly

All genome sequencing was performed at the Center for the Analysis of Genome Evolution and Function at the University of Toronto, Canada. Specifically, libraries were prepared using the Illumina Nextera Flex Library Preparation Kit, and sequencing was performed on an Illumina NextSeq2000 with a P1 300-cycle flow cell to produce 150bp paired-end reads for each strain. Raw sequencing reads were then quality trimmed, and adaptors were removed using Trim Galore (v0.6.10) [10]. Filtered reads were then assembled using SPAdes (v3.10.1) with the 'careful' parameter and *k*-mer lengths of 21, 33, 55, 77, 99 and 127 [11]. Contigs that were shorter than 500bp or had a *k*-mer coverage below 2.0 were removed from the assembly. Reads were then aligned back to the filtered contigs using Bowtie 2 (v2.3.3), and the resulting SAM files were converted to BAM format using SAMtools (v1.20) [12]. Final assemblies were then polished using Pilon (v1.24) [13] to improve the base call accuracy, and the completeness and contamination for each polished assembly were assessed using CheckM (v1.1.2) [14]. Finally, all genome statistics were calculated using a Python script available at: https://github.com/sujan8765/nepgorkhey_python/blob/master/genome_stats.py. Final genome assemblies were then deposited into the National Center for Biotechnology Information (NCBI) GenBank database under BioProject PRJNA1353131 and annotated using the NCBI Prokaryotic Genome Annotation Pipeline. All tools were run with default parameters unless stated otherwise.

Phylogenetic analysis

For the phylogenetic analyses, the six strains sequenced in this study were used in concert with a collection of type strains from each classified *Xanthomonas* species, along with additional strains from *X. perforans* (55), *X. euvesicatoria* (50) and *X. campestris* (50) (Table S1). The additional strains were selected as representatives of distinct phylogroups. Specifically, phylogenetic trees were first constructed using all available genome assemblies from the NCBI database for each species. Two to three representative strains from each phylogroup were then selected for the final phylogenetic trees displayed in the manuscript. All genomes were first reannotated using Bakta (v1.11.0) [15]. The resulting GFF3 files were then converted to GFF format to use as input for PIRATE (v1.0.5) [16], which enabled us to conduct a pangenome analysis on all strains and obtain a concatenated core-genome alignment. This concatenated core-genome alignment was then used as input for RAXML (v8.2.10) to construct a maximum likelihood phylogenetic tree using substitution model GTRGAMMAI with 500 bootstraps [17]. This specific pipeline was used four times in this study, once for the genus-wide tree to assign species designations to each of our newly sequenced strains and thrice for the *X. perforans*, *X. euvesicatoria* and *X. campestris* specific analyses. All final phylogenetic trees were then visualized and optimized using iTOL (v7) [18]. Average nucleotide identity (ANI) quantification of all six strains and the type strains of *X. perforans* (DSM 18975), *X. euvesicatoria* (LMG 27970) and *X. campestris* (ATCC 33913) was performed using pyANI plus (v1.0.0) with ANIb algorithm [19].

Identification of T3SEs and copper resistance genes

All T3SEs in each of our six genomes were identified using our in-house *Xanthomonas* T3SE database [20]. Specifically, a BLASTP (v2.15.0) analysis was performed using reference T3SEs as queries against the predicted proteins from each genome as subjects [21]. Hits with an *E*-value $<10^{-15}$ and sequence coverage $>40\%$ were considered present. The heatmap illustrating the presence-absence patterns of each T3SE family was generated using GENE-E (<https://software.broadinstitute.org/GENE-E/>).

Known copper resistance genes were also screened against the assembled genomes using BLASTN (v2.15.0) (percent identity $>90\%$, query coverage $>80\%$, *E*-value $<1e-5$) with the following reference genes: *X. perforans* strain GEV872 (*copL*, [WCC52262.1](#); *copA*, [WCC52263.1](#); *copB*, [WCC52264.1](#)); *X. euvesicatoria* strain LMG 930 (*copL*, [APO88841.1](#); *copA*, [APO88840.1](#); *copB*, [APO88839.1](#)); *X. campestris* strain CFBP 9145 (*copL*, [WMB27255.1](#); *copA*, [WMB27256.1](#); *copB*, [WMB27257.1](#)). While our prior analyses have suggested that it is relatively rare [20, 22, 23], it is worth noting that the presence of contig breaks and misassemblies in draft genomes, like the ones we present here, does have the potential to produce some false negatives and positives in this type of analysis.

RESULTS AND DISCUSSION

Genome sequencing and assembly

We assembled draft genome sequences of six new *Xanthomonas* strains from plants experiencing symptoms of bacterial spot (YA993, YA1076, YA1054, YA1087, YA1525 and YA1028) and deposited each assembly on NCBI. Accession numbers for each assembly are listed in Table 1. Additional metadata and assembly statistics associated with each strain are also summarized in Table 1. Specifically, the average genome size was ~ 5 Mbp, with the number of contigs ranging from 30 to 114. Sequencing coverage varied from 260x to 551x. The completeness of all new assembled genomes was estimated to be 99.64% and we found no evidence of contamination in any of the genomes that we are reporting.

Species-level identification of isolated strains

To assign a species-level designation to each of our newly isolated strains, we first conducted a pangenome analysis with our strains using a single representative genome from each established *Xanthomonas* species. This analysis enabled us to generate a concatenated core-genome alignment (comprising 2,063 core gene families) and generate a corresponding core-genome phylogenetic tree to evaluate the evolutionary relationships between our novel strains and each representative strain. Ultimately, we found that strains YA993, YA1076 and YA1054 clustered with *X. perforans*; strains YA1087 and YA1525 clustered with *X. euvesicatoria*; and strain YA1028 clustered with *X. campestris* (Fig. 1a). These species-level assignments were confirmed with pairwise ANI measurements, demonstrating that YA993, YA1076 and YA1054 all shared $\geq 99.9\%$ ANI with *X. perforans* reference genome DSM 18975; both YA1087 and YA1525 shared $\geq 99.7\%$ ANI with *X. euvesicatoria* reference genome LMG 27970; and YA1028 shared $\geq 98.7\%$ ANI with *X. campestris* reference genome ATCC 33913 (Fig. 1b).

Both *X. perforans* and *X. euvesicatoria* are well established causal agents of bacterial spot disease in tomato and pepper. Over the past three decades, *X. perforans* has emerged as the dominant tomato pathogen in the USA, particularly in Florida, displacing *X. euvesicatoria* [24]. Recent studies have also reported an expanded host range for *X. perforans* due to the shift from tomato to pepper, including the emergence of a new *X. perforans* lineage that has been isolated from pepper in the USA and Taiwan [25–27]. More recently, several *X. perforans* strains have also been isolated from pepper in Thailand, and a single strain was isolated from pepper in the Anatolia Region in 2020 [8]. Our isolation of *X. perforans* YA993, *X. perforans* YA1076, *X. euvesicatoria* YA1087 and *X. euvesicatoria* YA1525 from pepper in Adana and Mersin suggests that both of these pathogens are circulating in pepper

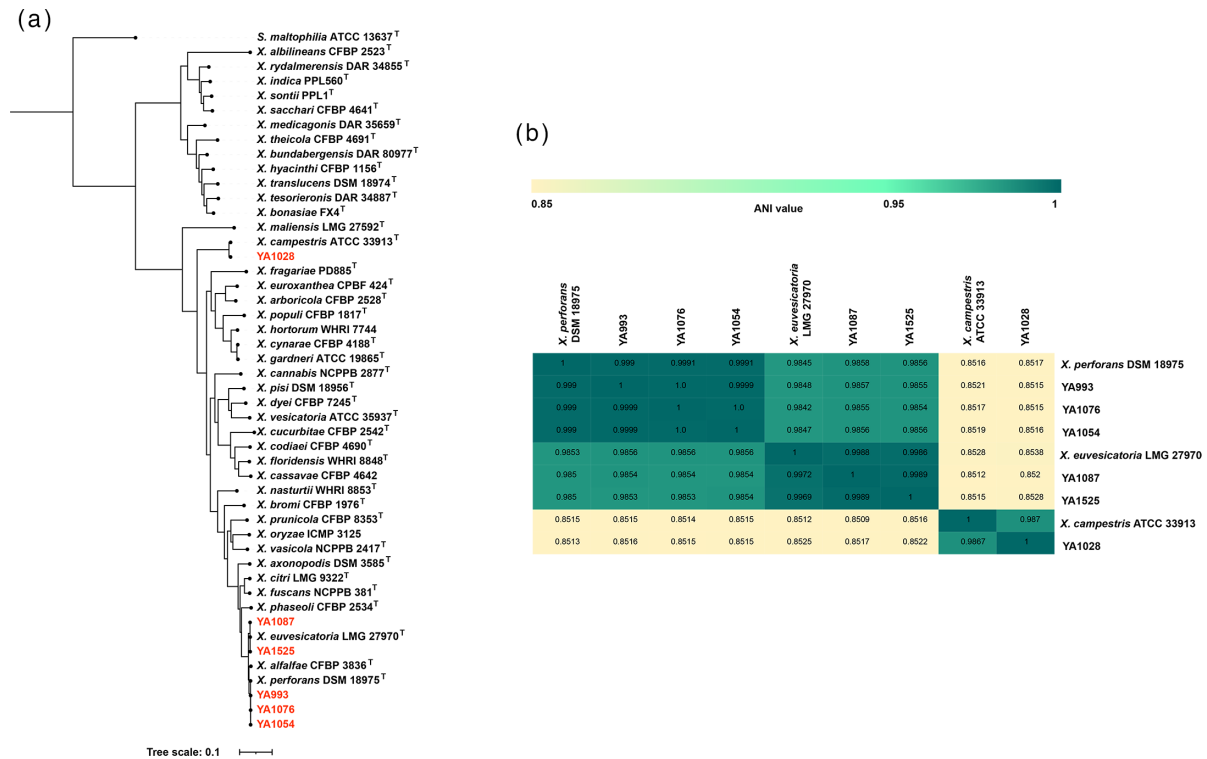


Fig. 1. (a) Core-genome phylogenetic tree placing the six newly sequenced *Xanthomonas* strains from this study in the context of representative strains from each established *Xanthomonas* species. The core-genome phylogenetic tree was constructed using RAXML and visualized using iTOL. *Xanthomonas* strains from this study are highlighted in red. The scale represents the number of substitutions per site. *Stenotrophomonas maltophilia* (ATCC 13637) was used as an outgroup to root the broader *Xanthomonas* tree. (b) Heatmap showing ANI comparisons between the six sequenced strains and the corresponding type strains to which they were assigned (*X. perforans* DSM 18975, *X. euvesicatoria* LMG 27970 and *X. campestris* ATCC 33913).

plants across Turkey. The fact that we also isolated *X. perforans* YA1054 from tomato further demonstrates that at least some of these strains are also likely capable of infecting tomato.

The assignment of strain YA1028 to *X. campestris* was more puzzling. Different pathovars of *X. campestris* exhibit different host specificities, with pv. *campestris* primarily causing black rot on cruciferous plants, pv. *raphani* primarily causing leaf spot on cruciferous and solanaceous plants and pv. *incanae* primarily causing bacterial blight on ornamental *Brassica* spp. [28]. Based on our phylogenetic analysis, the YA1028 strain is part of a monophyletic cluster that only includes pv. *campestris* strains. While it is certainly possible that this strain could cause disease in pepper, as has been observed previously in pv. *raphani* strains [29, 30], it could also be the case that YA1028 was not the causative agent of disease at all here. Consistent with this interpretation, an earlier pv. *campestris* strain that was isolated from a tomato greenhouse in Canada failed to induce typical bacterial spot symptoms on its own on either tomato or pepper [31]. Future studies will seek to test the pathogenicity of *X. campestris* YA1028 on pepper, though an important challenge here will be to recapitulate the environmental conditions in the field that led to the bacterial spot symptoms on this host.

Evolutionary relationships of strains within each species

To further refine the evolutionary context of the newly classified *X. perforans*, *X. euvesicatoria* and *X. campestris* strains isolated in this study, we also performed species-specific pangenome and core-genome phylogenetic analyses using at least 50 diverse genomes from each species. Genomes were chosen to represent the global diversity within each species based on their prior assignment to phylogenetic clusters, the hosts they were isolated from and the sites from which they were isolated (Table S1). A core-genome phylogenetic tree of each of our novel strains in the context of their assigned species ultimately helped us deduce where these strains likely came from and whether they represent new lineages of bacterial spot pathogens or were transmitted to these regions from known bacterial spot lineages.

For the three strains assigned to *X. perforans* (YA993, YA1054 and YA1076), we found that all strains clustered closely with recently reported *X. perforans* strains from Turkey that were isolated from both tomato and pepper (Fig. 2a). These Turkish strains were

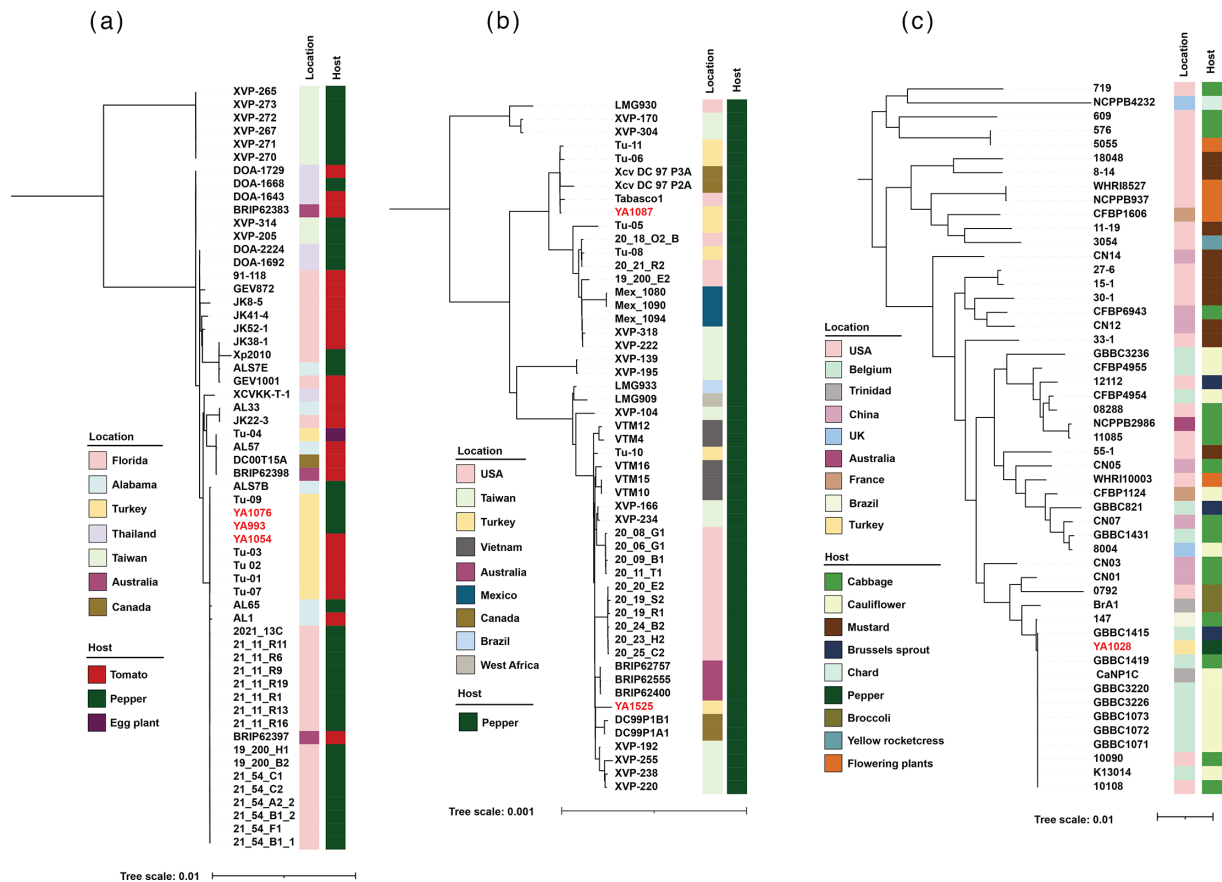


Fig. 2. Species-specific evolutionary relationships between the six strains isolated in this study and previously isolated strains of each species based on a core-genome phylogeny: (a) *X. perforans*, (b) *X. euvesicatoria* and (c) *X. campestris*. The phylogenetic trees were rooted at the midpoint, and the tree scale reflects the number of substitutions per site. Strains that were the focus of this study are highlighted in red.

collectively part of a larger clade that also includes *X. perforans* strains isolated from pepper and tomato in the USA, suggesting a shared evolutionary origin across distant geographic regions. Other *X. perforans* strains isolated from pepper, including Xp2010 from the USA; DOA-1668, DOA-2224 and DOA-1692 from Thailand; and XVP-205 and XVP-314 from Taiwan, were also closely related to strains isolated from tomato. This tendency of pepper and tomato infecting strains to be intermixed across the phylogenetic tree of *X. perforans*, particularly within clades of very similar strains, suggests that many of these strains are capable of infecting both hosts or that host switches have occurred many times in this lineage through relatively few genetic changes.

In contrast to the *X. perforans* strains, the two strains that we assigned to *X. euvesicatoria* (YA1087 and YA1525) belonged to two separate clades (Fig. 2b). Specifically, strain YA1087 was assigned to a clade with other previously reported *X. euvesicatoria* bacterial spot pathogens from Turkey, the USA and Canada. Similarly, YA1525 was also assigned to a broadly distributed clade of *X. euvesicatoria* bacterial spot pathogens that were isolated from pepper. This clade does include one other *X. euvesicatoria* strain that was isolated from Turkey back in 2020, but also includes strains isolated from Australia, Canada, Taiwan, the USA and Vietnam. These data suggest that there are likely multiple lineages of *X. euvesicatoria* circulating in Turkey that are causing bacterial spot disease and that both of these lineages are broadly distributed globally. The fact that the two strains were isolated from different locations (YA1087 from Adana and YA1525 from Mersin) may further indicate that distinct lineages are endemic to particular regions in Turkey. However, this will require further investigation through increased sampling in both Adana and Mersin, along with other pepper cultivating regions in Turkey.

Finally, our phylogenetic analysis in *X. campestris* revealed that *X. campestris* YA1028 clusters with several *X. campestris* pv. *campestris* (Xcc) strains isolated from cruciferous crops like cabbage, cauliflower, broccoli and brussels sprouts (Fig. 2c). The global distribution of these strains was also quite broad, including strains from Brazil, Belgium, Trinidad and the USA. These associated hosts and locations are consistent with what we know about Xcc [28], which supports the notion that it may not be a novel pathogenic bacterial spot pathogen on its own but just happened to be colonizing a pepper plant experiencing bacterial spot disease as a result of either the climatic conditions or because of co-infection with another pathogen. As suggested above,

future studies will aim to characterize the host range of this strain and assess the environmental and ecological conditions in which it causes disease in pepper.

Distribution of T3SEs in isolated strains

T3SEs are major virulence factors in most *Xanthomonas* species. In order to explore the diversity of T3SEs in our six newly sequenced *Xanthomonas* strains, we performed a BLAST analysis using an in-house database that contains a diverse collection of representative T3SEs from *Xanthomonas* (Fig. 3) [20]. A comparison of the T3SE repertoires of these six strains belonging to *X. perforans*, *X. euvesicatoria* and *X. campestris* revealed 22 core T3SE families that were present in all six strains from these three species. An additional 29 T3SE families were only present in one or two of the species analysed. Within species where we sequenced multiple strains, the three *X. perforans* strains had a fully conserved T3SE repertoire, which is consistent with the fact that these strains were closely related based on our core-genome analysis. On the other hand, *X. euvesicatoria* YA1525 harboured four additional T3SEs that were absent from *X. euvesicatoria* YA1087 (XopAF, XopAQ, XopAX and XopBB). This diversity in T3SE repertoire reflects the fact that these two strains come from distinct *X. euvesicatoria* lineages and may result in distinct host ranges for these strains [32].

In total, the three *X. perforans* strains harboured 33 T3SEs that were present across all strains. This is consistent with prior observations across a broader diversity of *X. perforans* strains, which harboured an average of 33.35 T3SEs per strain [20]. In addition to harbouring all 33 of the *X. perforans* T3SEs, the 2 *X. euvesicatoria* strains also harboured 7 additional T3SE families, with both *X. euvesicatoria* strains harbouring XopAA, XopAJ and XopB, and only *X. euvesicatoria* YA1525 harbouring XopAF, XopAQ, XopAX and XopBB. The fact that two distinct *X. euvesicatoria* lineages harbour larger T3SE repertoires than *X. perforans* may suggest that this lineage of bacterial spot pathogens has a slightly expanded T3SE repertoire, which could result in a narrower host range because of effector triggered immunity (ETI) [32]. Finally, the T3SE profile of *X. campestris* YA1028 is quite distinct from those of *X. perforans* and *X. euvesicatoria*, including a total of 33 T3SEs, 8 of which were not identified in either *X. perforans* or *X. euvesicatoria* (AvrBs1, AvrXccA, hrpW, XopA, XopAC, XopAG, XopAH, XopAL, XopAM, XopG and XopH). This distinct T3SE repertoire is consistent with the fact that *X. campestris* is not especially closely related to *X. perforans* and *X. euvesicatoria* within the *Xanthomonas* lineage. However, the fact that *X. campestris* strains tend to have slightly smaller T3SE repertoires than *X. perforans* and *X. euvesicatoria* suggests that this is quite a large T3SE repertoire by *X. campestris* standards [20]. The presence of XopAC and XopAM in *X. campestris* YA1028 was also notable, given that these are key host range determinants that can elicit ETI on *Arabidopsis thaliana* [33, 34].

While this still requires experimental verification, it is worth noting that XopAQ has previously been reported to be plasmid-encoded [35], suggesting a potential role for plasmids in the expansion of the *X. euvesicatoria* YA1525 T3SE repertoire. Indeed, *in silico* plasmid prediction using Mob-suite identified a plasmid in this strain predicted to carry XopAQ, XopAX and XopE3 [36]. This supports the hypothesis that plasmids have introduced at least some new T3SEs into the lineage harbouring this strain. No plasmid was predicted in *X. euvesicatoria* strain YA1087, and while a single plasmid was predicted in the three *X. perforans* strains and two plasmids were predicted in *X. campestris* YA1028, none of these plasmids was predicted to encode any T3SEs.

In addition to T3SE repertoires, copper resistance can play an important role in *Xanthomonas* pathogenicity due to the broad use of copper-based bactericides in bacterial spot disease control. In *Xanthomonas*, copper resistance is typically conferred by an operon containing *copL*, *copA* and *copB* (*copLAB*). Interestingly, while copper is a common bactericide that is used in agriculture around the world, none of the six novel strains that we sequenced in this study contained copper resistance genes. This may be the result of differential copper use in Turkish agriculture and suggests that many of the bacterial spot pathogens circulating in Turkey are copper-sensitive.

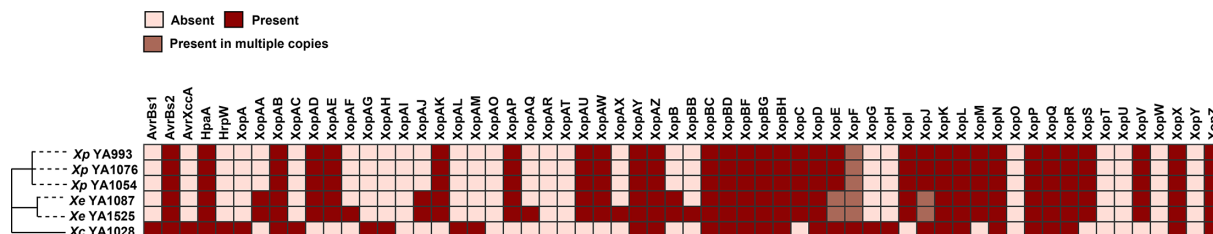


Fig. 3. Presence-absence map illustrating the T3SE repertoires of the six *Xanthomonas* strains sequenced in this study. T3SEs are shown along the top and strains are shown on the left, sorted using a phylogenetic tree based on the core-genome phylogeny. Homologs within the same family were grouped together under a single name (e.g. XopJ4, Xop J5=XopJ). Abbreviations: Xp, *X. perforans*; Xe, *X. euvesicatoria*; and Xc, *X. campestris*.

CONCLUSIONS

In this study, we sequenced six novel *Xanthomonas* strains isolated from recent outbreaks of bacterial spot in Turkey and analysed the evolutionary relationships between these strains and previously isolated *Xanthomonas* species. Specifically, we showed that the six novel strains were from *X. perforans* (3), *X. euvesicatoria* (2) and *X. campestris* (1). While the *X. perforans* strains were all part of the same evolutionary lineage, that was not the case for *X. euvesicatoria*, where strains from two distinct lineages with divergent T3SE repertoires were isolated from two distinct sites. Finally, while our *X. campestris* strain was isolated from a pepper plant infected with bacterial spot, we cannot be certain that this strain was the causative agent of disease on this host. These genomic data will be a valuable resource for understanding genetic diversity, host range and transmission of these *Xanthomonas* strains in Turkey and beyond.

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Author contributions

M.M.D. and Y.A. conceived and designed the study. Y.A. isolated *Xanthomonas* strains from diseased plants. M.M.D. stored the strains and prepared genomes for sequencing. A.K. assembled the genomes, conducted phylogenomic analyses and drafted the manuscript with inputs from M.M.D. A.K., M.M.D., E.M.G. and J.B.J. reviewed, edited and finalized the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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