Pathogenic and Comparative Genomic Analysis of *Ralstonia pseudosolanacearum* Isolated from *Casuarina*

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Abstract

Casuarina equisetifolia is crucial in protecting coastal regions of China against typhoon attacks but has faced a substantial challenge due to wilt disease caused by pathogens of the *Ralstonia solanacearum* species complex (RSSC). Although the initial outbreak of *Casuarina* wilt in the 1970s was effectively controlled by disease-resistant *C. equisetifolia* varieties, the disease has recently re-emerged in coastal regions of Guangdong. In this study, we report the isolation, characterization, and comparative genomic analysis of 11 RSSC strains from diseased *C. equisetifolia* at various locations along the coast of Guangdong. Phylogenomic analysis showed that the strains were closely related and clustered with phylotype I strains previously isolated from peanuts. Single-gene-based analysis further suggested these strains could be derived from strains present in Guangdong since the 1980s, indicating a historical context to their current pathogenicity. *Casuarina*-isolated strains exhibited notably higher virulence against *C. equisetifolia* and peanuts than the representative RSSC strains GMI1000 and EP1, suggesting host-specific adaptations that possibly contributed to the recent outbreak. Comparative genomic analysis among RSSC strains revealed a largely conserved genome structure and high levels of conservation in gene clusters encoding extracellular polysaccharide biosynthesis, secretion systems, and quorum sensing regulatory systems. However, we also found a number of unique genes in the *Casuarina*-isolated strains that were absent in GMI1000 and EP1, and vice versa, pointing to potential genetic factors underpinning their differential virulence. These unique genes offer promising targets for future functional studies. Overall, our findings provide crucial insights into the RSSC pathogens causing *Casuarina* wilt in Guangdong, guiding future efforts in disease control and prevention.

Keywords: bacterial wilt, *Casuarina equisetifolia*, comparative genomic, differential virulence, genetic variation, *Ralstonia pseudosolanacearum*

Ralstonia solanacearum species complex (RSSC) is a gram-negative bacterial pathogen capable of causing destructive infections in more than 450 plant species from more than 50 families (Jiang et al. 2017). It is recognized as one of the top 10 most important bacterial plant pathogens in the world (Mansfield et al. 2012), largely because of its remarkable ability to infect a broad range of host plants and to survive in diverse geographical regions. The RSSC can cause wilt diseases in various plants, including dicotyledonous herbs such as Solanaceae and Leguminosae, dicotyledonous woody plants such as mulberry and eucalyptus, as well as monocotyledons such as banana and ginger (Xu et al. 2009). Bacterial wilt diseases are prevalent not only in tropical and subtropical regions but also in temperate and cold areas (Jiang et al. 2017; Liu et al. 2016). A growing number of studies have shown that strains isolated from different regions or hosts exhibit variations in pathogenicity, host ranges, and genome compositions (Buddenhagen and Kelman 1964; Li et al. 2016). Consequently, the pathogen is often referred to as the RSSC (Balamurugan et al. 2020; K. Chen et al. 2021; Fegan and Prior 2005).

In order to differentiate various RSSC strains, several subdividing classifications, such as phylotypes, biovars, races, and sequevars, have been proposed based on different criteria. Based on the 16S-26S internal transcribed spacer (ITS) region, *egl* gene, *hrpB* gene, and *mutS* gene, Fegan and Prior (2005) proposed a classification scheme in which strains were divided into four phylotypes, each associated with different geographical regions. Each phylotype was further divided into different sequevars based on the *egl* gene (Balamurugan et al. 2020; Fegan and Prior 2005; Meng 2013; Poussier et al. 2002). Recent studies have proposed to reclassify RSSC strains into three species, namely, *R. pseudosolanacearum* (phylotypes I and III), *R. solanacearum* (phylotype II), and *R. syzygii* (phylotype IV), based on phylogenetic analysis of the 16S-23S rRNA ITS gene sequence, 16S–23S rRNA intergenic spacer (ITS) region sequences, partial *egl* gene sequence, proteomic data, and DNA–DNA hybridization data (Prior et al. 2016; Safni et al. 2014).

China, with its coastline spanning more than 32,000 km, frequently experiences typhoon attacks, especially in the southern and eastern coastal regions, severely impacting the local population, property safety, and ecological environment. In this regard, *Casuarina equisetifolia* (commonly known as ironwood; hereafter referred to as *Casuarina*) coastal forest shelterbelts play an important role in mitigating typhoon damages. *Casuarina* is an economically and ecologically important coastal afforestation species with more than 2 million hectares cultivated globally. Native to Southeast Asia, Malaysia, Australia, and other Oceania countries (Ayin et al. 2019; Potgieter et al. 2014a, b), *Casuarina* was introduced to the tropical and subtropical

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Data availability: Original high-throughput sequencing data and de novo genome assemblies of all *Casuarina* and *Casuarina*-isolated strains reported in this study have been deposited in the NCBI database under the BioProject accession PRJNA1037066 (in processing). Genome assemblies of all *Casuarina* isolates reported in this study have also been deposited in the Figshare database under the website: https://figshare.com/s/4b0d6b54a2200e603e2d.

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zones of China in 1897 for windbreaks and wood production (Zhang et al. 2020; Zhong et al. 2010). Presently, it has become an important shelter forest tree species in China that occupies approximately 300,000 ha, mainly in the Guangdong, Guangxi, Fujian, and Hainan provinces (Sun et al. 2014; Zhong et al. 2010, 2019).

Casuarina wilt, a soilborne disease caused by RSSC pathogens, was first reported by Orian G (1961) on *Casuarina* seedlings in Mauritius (Ayin et al. 2015, 2019). Later, *Casuarina* wilt was also found in Guangdong Province, China, from 1969 to 1985, where the outbreak of this disease caused massive damage to the *Casuarina* coastal forest shelterbelts (Guo and Liang 1986; Peng 2000). Characterization of the pathogens from various regions of Guangdong showed that the pathogenic strains belong to RSSC phylotype I, mostly biovar 3 and some biovar 4 (Liang and Chen 1984). The epidemic spread of *Casuarina* wilt was controlled by selection and large-scale plantation of disease-resistant clones of *Casuarina* in the 1980s (Peng 2000). However, an outbreak of *Casuarina* wilt occurred again recently in the coastal region of Guangdong Province, causing massive death of *Casuarina* trees. The reason for the sudden susceptibility of the previously wilt-resistant *Casuarina* trees to the RSSC is unclear.

To investigate this, in this study, we isolated 11 RSSC strains from the *Casuarina* trees showing typical wilt symptoms in several different coastal regions of Guangdong. We characterized the pathogenicity and genome sequences of these *Casuarina*-isolated RSSC strains and conducted comparative genomic analyses between these strains and those isolated from other hosts. We also explored the phylogenetic diversity of *Casuarina*-associated RSSC strains isolated in this and previous studies and examined among-strain variations in key virulence genes and pathogenic regulatory systems. The data and findings reported in this study offer valuable resources and insights for understanding the epidemiology and pathogenic mechanisms behind the *Casuarina* wilt outbreak.

Materials and Methods

Sample collection, bacterial isolation, and molecular testing

C. equisetifolia samples were collected from seven places in Guangdong Province of China, including Maoming, Shanwei, Yangjiang, and Zhanjiang regions, in October 2018. Samples were collected from *C. equisetifolia* trees with or without wilt symptoms. The tree samples were cut into 4- to 5-cm segments, sterilized in 95% alcohol for 30 s, disinfected the alcohol-treated samples over flame, and then cleaned two times with sterilized water. The samples were placed in a centrifuge tube containing 15 ml of sterilized water with gentle shaking for 5 h before spreading onto the plates containing YEB medium (per liter contains 10 g of tryptone, 5 g of yeast extract, 5 g of KCl, 5 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 15 g of agar powder, with pH 7.0 to 7.2) at 28°C for about 48 h. The single colonies were purified again by striking on a YEB plate, and bacterial stocks were prepared by inoculation of single colony in TTC liquid medium (per liter contains 10 g of tryptone, 5 g of glucose, 1 g of acid hydrolyzed casein, 0.5 g of 2, 3, 5-triphenyltetrazolium chloride, and 15 g of agar powder, with pH 7.0 to 7.2) and cultured for 24 h at 28°C with shaking at 200 rpm. The *Casuarina*-isolated bacterial strains from this study are listed in Table 1.

The *Casuarina*-isolated strains were characterized by two rounds of PCR analysis. First, PCR amplification was performed using RSSC-specific primers (759: 5′-GTCGCCGTCAACTCACTTTCC-3′; 760: 5′-GTCGCCGTCAGCAATGCGGAATCG-3′) (Fegan and Prior 2005). Second, PCR was carried out with the 16S universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-GGTTACCTTGTTACGACTT-3′). The PCR products were sent for DNA sequencing analysis after gel electrophoresis, and the sequence data were searched against the NCBI 16S rRNA database.

Genome sequencing, assembly, and annotation

Each of the 11 strains was cultured in liquid YEB medium, and genomic DNA was extracted using EasyPure Bacteria Genomic DNA Kit (Transgen, Beijing, China). The harvested DNA was detected by agarose gel electrophoresis and quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Fragmented genomic DNA with an average size of 300 bp and long DNA fragments of 23 kbp were selected for library preparation and sequenced on BGISEQ-500 and Nanopore PromethION platforms, respectively. For each of the 11 sequenced strains, a draft genome assembly was first obtained from the ONT sequencing data alone using Flye 2.9, and the draft was polished with both BGI and ONT sequencing data using NextPolish version 1.4. Genome annotations of all strains were generated using PGAP version 2022-02-10 (build 5872).

Sequevar analyses

For sequevar analysis, the *egl* gene sequence was obtained from each of the 11 *Casuarina*-isolated strains through PCR amplification (Endo-F: 5′-ATGCATGCCGCTGGTCGCCGC-3′; Endo-R: 5′-GCGTTGCCCGGCACGAACACC-3′) and Sanger sequencing. The *egl* gene sequences of 107 other RSSC strains with known sequevar information were obtained from the *R. solanacearum* species complex *egl* reference database (https://dataverse.cirad.fr/dataset.xhtml?persistentId=doi:10.18167/DVN1/CUWA5P). A phylogenetic analysis of the *egl* sequences was conducted following the protocol developed by Cellier et al. (2023).

Pathogenicity assay

In this experiment, the root dip method was used to determine the pathogenicity of the isolated strains. Each strain was streaked on a TTC plate and incubated at 28°C in an incubator for 48 h, followed by picking a single colony from TTC liquid medium for overnight culture, then inoculating it into 100 ml of TTC liquid medium, and placing it in a shaker at 28°C. After 24 h, the concentration was adjusted to an OD_{600} value of 0.8 with sterile water. About one-third of the roots of *Casuarina* seedlings about 40 to 50 cm in height and other plants (tomato, peanut, and tobacco) about 20 to 30 cm in height were cut off to generate wounds, which were then soaked in the containers with bacterial solution for 20 min (other hosts) or 40 min (*Casuarina* seedlings). The treated seedlings were potted and placed in an incubator at 28°C. Each experiment was repeated three times, each time with seven *Casuarina* seedlings (or five seedlings for peanut, tomato, and tobacco) per isolate. The disease symptoms were observed daily, and severities were recorded according to the criteria listed in Supplementary Table S10. The disease index (DI) was calculated according to Supplementary Table S10. The survival curve was based on the first occurrence of *Casuarina* seedlings. When more than 50% of the twigs fell, it was regarded as death and marked as 1; when less than 50% of the twigs fell, it was marked as 0. The survival of each day was counted in turn, and finally, the average value of three experiments was taken to plot.

Phylogenetic and comparative genomic analyses

To investigate the phylogenetic relationship between the 11 *Casuarina*-isolated strains and other RSSC strains, the genome sequences of 25 representative RSSC strains were downloaded from the NCBI RefSeq database (Supplementary Table S3), including 20, 3, 1, and 1 strains from phylotypes I, II, III, and IV, respectively.

Table 1. Summary of the 11 *Casuarina*-isolated *Ralstonia solanacearum* species complex strains reported in this study

Strains	Host plant	Origin	Phylotype
MMB5	*C. equisetifolia*	Dianbai, Maoming City	I
MMB7	*C. equisetifolia*	Dianbai, Maoming City	I
SWA6	*C. equisetifolia*	Lufeng, Shanwei City	I
SWC4	*C. equisetifolia*	Lufeng, Shanwei City	I
YJA2	*C. equisetifolia*	Hailing Island, Yangjiang City	I
XWI2	*C. equisetifolia*	Xuwen, Zhanjiang City	I
XWJ3	*C. equisetifolia*	Xuwen, Zhanjiang City	I
WYF3Z	*C. equisetifolia*	Wuyang, Zhanjiang City	I
WYG1S	*C. equisetifolia*	Wuyang, Zhanjiang City	I
DHD47	*C. equisetifolia*	Donghai Island, Zhanjiang City	I
NS25	*C. equisetifolia*	Nansan Island, Zhanjiang City	I

A genome-based phylogenetic tree of the 36 genomes was then constructed using tANI_tool version 1.3.0 (Gosselin et al. 2022), and its reliability was evaluated with 100 bootstrap replicates.

For single-gene-based analysis, a total of 116 nucleotide sequences representing 12 genes (*16S rRNA*, *adk*, *dnaA*, *egl*, *gap*, *gdhA*, *gyrB*, *hrpB*, *leuS*, *mutS*, *rplB*, and *RipTALI-1*) from 31 RSSC strains isolated from *C. equisetifolia* were retrieved from the NCBI GenBank database (Supplementary Table S6). The 116 nucleotide sequences were searched against genome sequences of the 36 RSSC strains abovementioned using BLASTN version 2.14.0. Identification of orthologous gene groups was conducted for EP1, GMI1000, and the 11 *Casuarina*-isolated strains using OrthoFinder version 2.5.2 (Emms and Kelly 2019). The flower plot and Venn diagram for the visualization of presence or absence of patterns of orthologous genes were created using EVenn (http://www.ehbio.com/test/venn) (T. Chen et al. 2021). Multiple genome alignments of RSSC strains were generated and visualized using progressiveMauve version 2.4.0 (Darling et al. 2010). Prophage sequences were identified in RSSC genomes using the PHASTER server (http://phaster.ca/) (Arndt et al. 2016).

Secondary metabolite biosynthetic gene clusters were predicted in the genomes of EP1, GMI1000, and the 11 *Casuarina*-isolated strains using antiSMASH version 6.0 (Blin et al. 2019). Gene clusters encoding type II, III, IV, and VI secretion systems were identified using TXSScan version 1.1.1 (Abby et al. 2016). Genes encoding type III effectors were identified using the Ralsto_T3E webserver (https://iant.toulouse.inra.fr/bacteria/annotation/site/prj/T3Ev3/) (Peeters et al. 2013). The extracellular polysaccharide (EPS) biosynthesis gene cluster and genes encoding the Phc, Sol, and Ras QS systems were identified through the BLASTP search using the corresponding protein sequences of the strain GMI1000 as queries. The mapping URL used for the gene cluster map in this study is drawn using ChiPlot (https://www.chiplot.online/) (Ji et al. 2022).

Results

Casuarina strains belong to RSSC phylotype I and different sequevars

Diseased *Casuarina* samples were collected at multiple locations spanning c.a. 1,000 km in distance along the coastal line of Guangdong, China, and the bacterial cells were isolated using YEB medium. According to the relative size of the RSSC hazard area and severity of wilt disease, 11 bacterial strains from seven regions, including Lufeng (two strains), Hailing Island (one strain), Dianbai (two strains), Wuyang (two strains), Donghai Island (one strain), Nansan Island (one strain), and Xuwen (two strains) were selected for further analysis (Table 1; Supplementary Fig. S1C). The PCR assay using RSSC-specific primer and subsequent gel electrophoresis showed that all the *Casuarina* bacterial strains produced a typical DNA band similar to the well-characterized RSSC strains GMI1000 and EP1. 16S rRNA sequencing analysis was also conducted, and the best matches were all from *R.* (*pseudo*)*solanacearum*, suggesting that the 11 *Casuarina*-isolated strains belong to the RSSC.

We then conducted phylotype and sequevar analyses. According to the Fegan and Prior method (Fegan and Prior 2005), the 11 RSSC strains belong to phylotype I (Table 1). The *egl* gene-based phylogenetic analysis (Balamurugan et al. 2020; Fegan and Prior 2005; Meng 2013) showed that strains DHD47, MMB5, MMB7, NS25, WYF3Z, WYG1S, XWJ3, and YJA2 are closely related to Sequevars 14 and 68, whereas strains SWA6, XWI2, and SWC4 belong to Sequevars 13, 17, and 44, respectively (Table 1; Supplementary Fig. S2).

Casuarina-isolated strains are virulent against *C. equisetifolia* seedlings and other hosts

The 11 *Casuarina*-isolated strains were examined for their virulence against *Casuarina*. Two RSSC phylotype I strains, GMI1000 and EP1, which were isolated from tomato and eggplant, respectively, were included in the pathogenicity test for comparison. In the *Casuarina* seedlings inoculated with *Casuarina*-isolated strains, wilting symptoms began to appear at 2 days postinoculation (dpi), with cladophyll dropping off, and these symptoms became very severe at 6 dpi and stabilized at 10 dpi. To measure the virulence of strains, we calculated the DI according to the relative disease severity at 14 dpi (Supplementary Table S10). Compared with strains GMI1000 and EP1, which caused minor infections on *Casuarina* seedlings with DI at 0.14 and 0.30, respectively, all the 11 newly isolated strains were virulent against their native host with the DI in the range between 0.5 and 0.9. In particular, the strains NS25 and YJA2 were the most virulent, with a DI higher than 0.8 (Fig. 1A and B). Ordinary one-way ANOVA test showed that all strains except EP1 exhibited a significantly greater DI than GMI1000 (Fig. 1A; P value = 0.9680 for EP1 and P value <0.0169 for all *Casuarina*-isolated strains). The same trend was revealed by analyzing the survival rate at 14 dpi; inoculation of the *Casuarina*-isolated strains resulted in survival rates of about 20 to 50%, while much higher survival rates were observed for GMI1000 and EP1 (>80%) (Supplementary Tables S11 and S12). All 11 strains isolated from *Casuarina* were highly virulent to *Casuarina*, while the reference strains GMI1000 and EP1, which were isolated from tomato and eggplant, respectively, showed lower virulence to *Casuarina*.

In addition, we compared the virulence of selected *Casuarina*-isolated strains (NS25, SWA6, SWC4, and YJA2), GMI1000, and EP1 on three other hosts, including peanut, tomato, and tobacco. The DI was calculated for each strain and host based on the relative disease severity at 10 dpi. The results showed that, on tomato and tobacco, all six strains tested were fully virulent with DI at 1. In comparison, the strains exhibited differential virulence on peanut; the *Casuarina*-isolated strains were highly virulent (DI = 1), while GMI1000 was avirulent and EP1 had intermediate virulence (DI = 0.4) (Supplementary Fig. S3).

Genome sequencing and analysis of *Casuarina*-isolated strains

Whole-genome sequencing of the 11 *Casuarina*-isolated RSSC strains was conducted with a combination of second- and third-generation high-throughput sequencing technologies, generating 1.52 to 3.55 Gb of BGI short-read data and 1.80 to 9.46 Gb of third-generation long-read data, respectively (Supplementary Table S1 and S2). All strains were successfully assembled into complete genomes consisting of two large circular replicons, commonly referred to as the chromosome (3.71 to 3.78 Mb) and megaplasmid (1.81 to 1.99 Mb), respectively. Except for strains SWA6, DHD47, and XWJ3, the other eight strains also have one to three small plasmids (1.7 to 129.6 Kb; Table 2). The total genome sizes of the strains range between 5.61 and 5.86 Mb, and the numbers of protein-coding genes range between 4,772 and 4,955, both smaller than most phylotype I RSSC genomes available in the NCBI RefSeq database (median genome size: 5.86 Mb; median protein-coding gene count: 5,023) (Table 2). In addition, the 11 *Casuarina* strains have 56 to 60 tRNA genes and 12 rRNA genes, except for XWI2, which has nine rRNA genes. The GC content of these strains is about 67%, highly similar to other phylotype I RSSC genomes (Table 2).

Genome-based phylogenetic analysis of *Casuarina*-isolated RSSC strains

To determine the evolutionary placements of the 11 *Casuarina*-isolated strains within RSSC, we conducted a whole genome-based phylogenetic analysis of these strains and 25 other RSSC strains representing the four phylotypes (Supplementary Table S3). The phylogenetic tree showed that the 11 *Casuarina* strains isolated in this study were all classified as phylotype I, the same as the three previously reported *Casuarina* strains (YQ, NCPPB 253, and Lallmahomed 59) (Fig. 2A). Interestingly, the two *Casuarina*-isolated strains collected from Africa (NCPPB 253 and Lallmahomed 59) and those collected from China (YQ and the 11 strains reported in this study) belong to two different divisions of phylotype I, respectively. Among the *Casuarina*-isolated strains from China, the 11 strains collected from Guangdong Province shared closer evolutionary relationships with each other than with the strain YQ, which was collected from Zhejiang Province.

Notably, the tree revealed a clade of 14 closely related strains, including 9 of the 11 *Casuarina*-isolated strains isolated in this study (DHD47, MMB5, MMB7, NS25, WYF3Z, WYG1S, XWI2, XWJ3, and YJA2), four strains isolated from peanut (362200, HA4-1, PeaFJ1, and Pn33), and one strain isolated from bitter melon (Bg07). These nine *Casuarina*-isolated strains in this clade were all collected from western Guangdong (Maoming, Yangjiang, and Zhanjiang cities), whereas the remaining two strains (SWC4 and SWA6) were collected from eastern Guangdong (Shanwei City). SWC4 was sister to this clade, while the strain SWA6 was relatively more divergent (Fig. 2A). A closer examination of the clade further indicates a monophyletic group of seven *Casuarina*-isolated strains collected from various locations in western Guangdong, while the remaining two strains (XWI2 and YJA2) both clustered with strains isolated from peanut (Fig. 2A).

Single gene-based analysis of the diversity of *Casuarina*-isolated RSSC strains

In addition to the three RSSC *Casuarina*-isolated strains (NCPPB 253, Lallmahomed 59, and YQ) with genome sequences available in the NCBI RefSeq database, a few dozen RSSC strains isolated from *Casuarina* have been partially sequenced. A search of the NCBI GenBank database identified 116-nucleotide sequences representing 12 single genes (e.g., *dnaA*, *egl*, *gap*, *gyrB*, and *hrpB*) from 31 *Casuarina*-associated RSSC strains isolated from China, Guam, and Mauritius (Supplementary Table S6). To further investigate the relationship between these previously isolated *Casuarina* strains and other RSSC strains, we conducted a BLASTN search of these single-gene sequences against the genome sequences of the three representative *Casuarina*-isolated strains from this study and another 28 representative RSSC strains and found (near-)perfect matches in

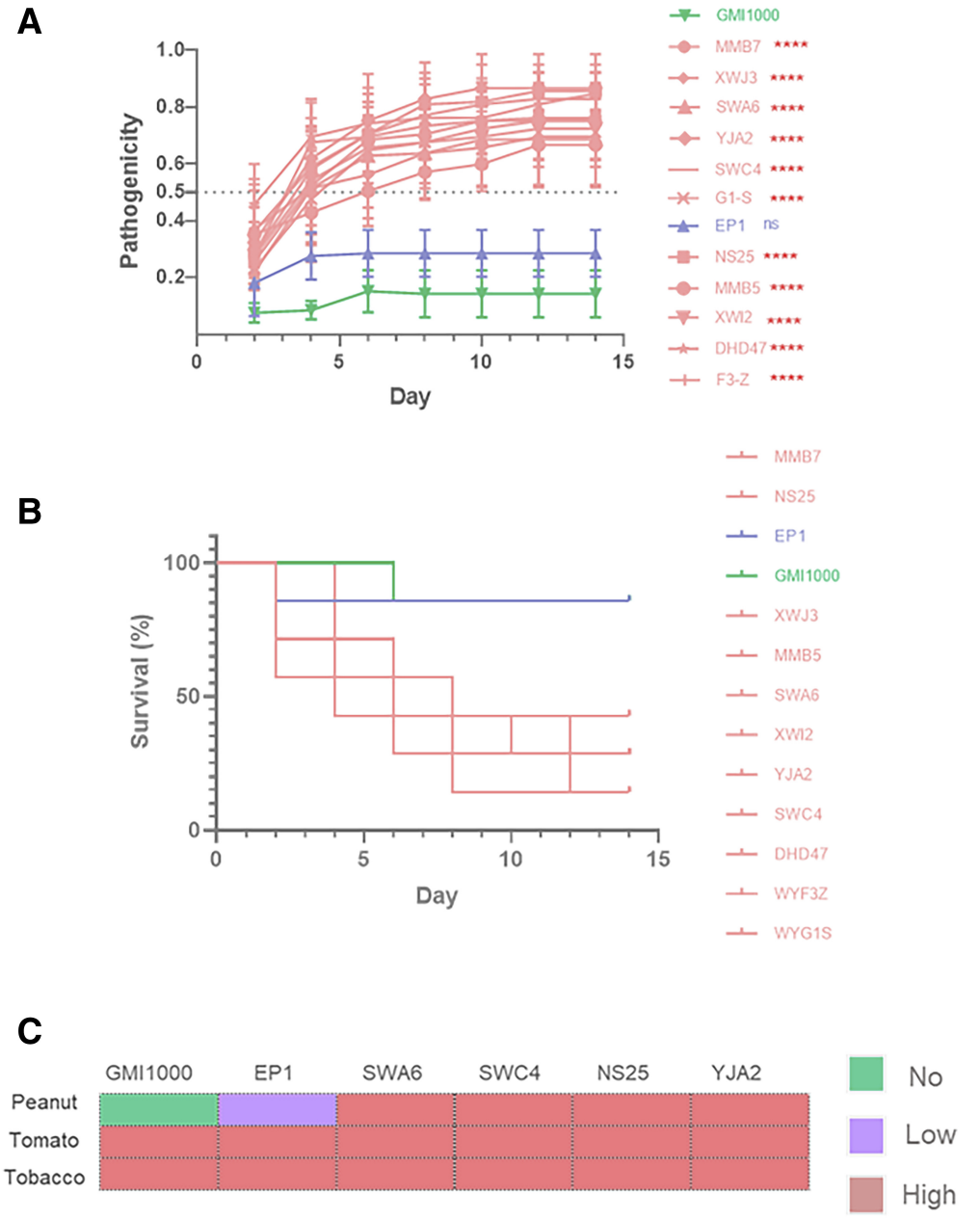

Fig. 1. Virulence of the 11 *Casuarina*-isolated strains and two representative *Ralstonia solanacearum* species complex (RSSC) strains, GMI1000 and EP1, on *Casuarina equisetifolia* and other hosts. **A,** Disease index curves of different RSSC strains on *Casuarina* from 2 to 14 dpi. **B,** Survival rate curves of different RSSC strains on *Casuarina* from 2 to 14 dpi. **C,** Virulence of representative strains on three other hosts, including peanuts, tomatoes, and tobacco. In the analysis of the disease index (A), GMI1000 which exhibited the lowest level of virulence was set as the reference in comparison with all other strains. Data in A and B are the means of three independent repeats, and error bars in A are standard errors. Statistical significance: ****P value < 0.0001 (ordinary one-way ANOVA).

one or more genomes for each of these single-gene sequences (Fig. 3; Supplementary Table S6). By taking all genes of the same strain into account, these 31 strains could be assigned to a specific clade in the RSSC phylogeny.

Importantly, two of the three previously characterized *Casuarina* strains from China, namely, GD1986C2 and China-Ironwood, matched specifically with the abovementioned clade containing nine of the *Casuarina*-isolated strains from this study (Fig. 3). While the exact location and date of isolation are unavailable for China-Ironwood, the GenBank record HM775340.1 indicates that GD1986C2 was collected from Guangdong in 1986. Therefore, our results suggest that the main RSSC lineage contributing to the current outbreak of Casuarina bacterial wilt in Guangdong has been present in the same region at least since 1986. Among the other strains, most Guam strains and the Mauritius strain showed best matches with the clade containing NCPPB 253 and Lallmahomed 59 (from Africa), and four Guam strains (A6407, A6413, 19-170, and 19-174-1) matched preferentially with the clade containing strain YQ (from Zhejiang, China), whereas four other Guam strains (A3450, A6124, A6364, and A6401) likely belong to phylotype II. Overall, our single-gene analysis further revealed the phylogenetic diversity of *Casuarina*-associated RSSC strains, which belong to at least three lineages in phylotype I and one lineage in phylotype II.

Genome comparison of *Casuarina*-isolated strains with EP1 and GMI1000

As revealed by our pathogenicity assays, the 11 *Casuarina*-isolated strains and two representative RSSC strains (EP1 and GMI1000) displayed contrasting virulence against *C. equisetifolia* and peanut (Supplementary Fig. S3). We therefore conducted a comparative genomic analysis of the strains to understand the molecular basis of the difference in their virulence. The results showed that all the 11 *Casuarina*-isolated strains share a core set of 4,110 orthologous gene groups. At the same time, the strains also have varying numbers of strain-specific genes; the most distantly related strains SWA6 and SWC4 have 177 and 107 strain-specific genes, respectively, while the seven closely related strains only have 0 to 2 (Fig. 2B). The vast majority of the 3,996 shared genes are also present in EP1 and/or GMI1000 (Fig. 2C). Importantly, there are 32 genes common to all 11 *Casuarina*-isolated strains but absent in EP1 and GMI1000, including those encoding transporter, GTP 3′,8-cyclase MoaA, FAD-dependent oxidoreductase, immunity-related proteins (probably antitoxins for T6SS toxins; Aoun et al. 2023), transcriptional regulators, and hypothetical proteins (Supplementary Table S4). Additionally, 98 genes are shared by EP1 and GMI1000 but absent in all 11 *Casuarina*-isolated strains, including many genes predicted to have functions related to mobile genetic elements (e.g., phage proteins) and the ABC transporter permease (Supplementary Table S5). These genes with contrasting presence or absence patterns in the 11 *Casuarina*-isolated strains and EP1/GMI1000 are promising candidates for future functional investigation.

We further carried out whole-genome alignment analysis for the RSSC strains and found that their genome structures were overall well-conserved. A strong collinearity was observed not only among the 11 *Casuarina*-isolated strains but also between them and other RSSC strains (data not shown). Most structural variations were small-scale translocation/inversion events, and only three relatively large-scale inversions were identified, including one on the chromosome in GMI1000 (Fig. 4A) and two on the megaplasmid in SWA6 and SWC4 (Fig. 4B). Notably, the structural variation hotspots (e.g., the regions around 0.2 and 2.80 Mb on the chromosome) largely overlapped with prophage sequences identified in the genomes (Fig. 4; Supplementary Table S7).

The whole-genome alignment analysis also revealed that the plasmids found in our study can be classified into three types. The most prevalent plasmid is present in 8 of the 11 *Casuarina*-isolated strains and two closely related peanut-isolated strains (HA4-1 and 362200, isolated from Hubei and Fujian provinces of China, respectively). This plasmid displayed considerable variations in size (87.55 to 143.76 kb) and arrangement among strains (data not shown). The other two plasmids are of much smaller sizes (2.99 and 1.74 kb) and are found in only three and two strains, respectively; although their sequences are highly conserved, these very small plasmids might represent assembly artefact and thus should be interpreted with caution.

Secondary metabolite biosynthetic gene cluster: RSSC strains can produce a variety of secondary metabolites that play important roles in bacterial virulence (e.g., ralfuranones), competition with other microorganisms (e.g., micacocidin), and interkingdom communication with fungi (e.g., ralsolamycin). A total of 12 secondary metabolite biosynthetic gene clusters (SMBGCs) were predicted in the examined RSSC strains, including four T1PKS/NRPS clusters, two aryl polyene clusters, two HSL clusters, one terpene cluster, one furan cluster, one NI-siderophore cluster, and one RIPP-like cluster. Four of the T1PKP/NRPS clusters are known SMBGCs producing micacocidin, ralfuranone, ralsolamycin, and rhizomide, respectively (Supplementary Table S8). The same set of SMBGCs was found in all strains except for SWC4, which lost the ralsolamycin-producing T1PKS/NRPS cluster, and their genomic locations are highly conserved as well (Fig. 5A; Supplementary Table S8). The results suggest that the phylotype I RSSC strains are highly similar in their secondary metabolite biosynthesis capabilities.

EPS biosynthesis gene cluster: EPSs play a pivotal role in various biological processes of RSSC such as biofilm formation, thereby significantly influencing the pathogenicity and survival strategies of the pathogen in diverse ecological niches. The production of EPS I in the RSSC requires the *eps* gene cluster consisting of seven genes (*epsAPBCDEF*) (Huang and Schell 1995). The analysis showed that

Table 2. Genomic characteristics of the 11 *Casuarina*-isolated *Ralstonia solanacearum* species complex strains[a]

Strains	Accession	Contig number			Genome size (nt)	G + C (%)	CDS	rRNA	tRNA	Pseudogene	Transposase
		Chromosome	Megaplasmid	Plasmid							
GMI1000	GCF_000009125.1	1	1	0	5,810,922	67	4,991	12	59	122	139
EP1	GCF_002215585.1	1	1	0	6,042,968	67	5,228	12	60	108	123
MMB5	In processing	1	1	1	5,849,427	67	4,955	12	58	134	176
MMB7	In processing	1	1	2	5,850,254	67	4,943	12	58	134	178
NS25	In processing	1	1	1	5,843,645	67	4,949	12	57	136	185
SWC4	In processing	1	1	1	5,612,821	67	4,772	12	58	102	129
WYF3Z	In processing	1	1	2	5,855,909	67	4,953	12	58	132	180
WYG1S	In processing	1	1	2	5,844,861	67	4,953	12	57	131	182
XWI2	In processing	1	1	1	5,609,760	67	4,795	9	56	129	169
XWJ3	In processing	1	1	0	5,753,119	67	4,870	12	58	132	171
YJA2	In processing	1	1	2	5,839,869	67	4,954	12	58	131	172
DHD47	In processing	1	1	0	5,770,586	67	4,879	12	57	128	174
SWA6	In processing	1	1	0	5,704,821	67	4,872	12	60	110	89

[a] Two representative *Ralstonia solanacearum* species complex strains, GMI1000 and EP1, were included for comparison.

the *eps* gene cluster is highly conserved in all strains examined (Fig. 5B).

Type II secretion system: The type II secretion system (T2SS) transports various proteins from intracellular to extracellular and plays a role in the disease process. In the RSSC, T2SS is encoded by the *gsp* gene cluster consisting of more than 12 genes (*gspC-N*) (da Silva Xavier et al. 2022). Our results showed that the *gsp* cluster was present and highly conserved in all examined strains; all *Casuarina*-isolated strains have the same set of genes in the *gsp* cluster, and all the gene sequences are highly similar to their respective orthologs in GMI1000 (>99% protein sequence similarity, 100% coverage) (Supplementary Fig. S4A).

Type III secretion system: Type III secretion system (T3SS) is an important and conserved class of virulence-related genes present in many pathogenic bacteria. It can secrete Type III secreted effectors (T3SEs), which facilitate pathogen–host interaction and promote the pathogen to obtain nutrition or inhibit the defense response of the host plant (Poueymiro and Genin 2009). Studies have shown that T3SS is related to host specificity (Pensec et al. 2015). In the RSSC, T3SS is encoded by the *hrp* gene cluster consisting of more than 20 genes and is about 30 kb in size (Genin and Boucher 2004). Our results showed that the *hrp* cluster was present and highly conserved in all examined strains. All *Casuarina*-isolated strains have the same set of genes in the *hrp* gene cluster, and all the gene sequences are highly similar to their respective orthologs in GMI1000 (>99% protein sequence similarity, 100% coverage). The only exception is the *popC* (*RipAC*) gene, which is predicted to be functional in GMI1000 and EP1 but annotated as a pseudogene

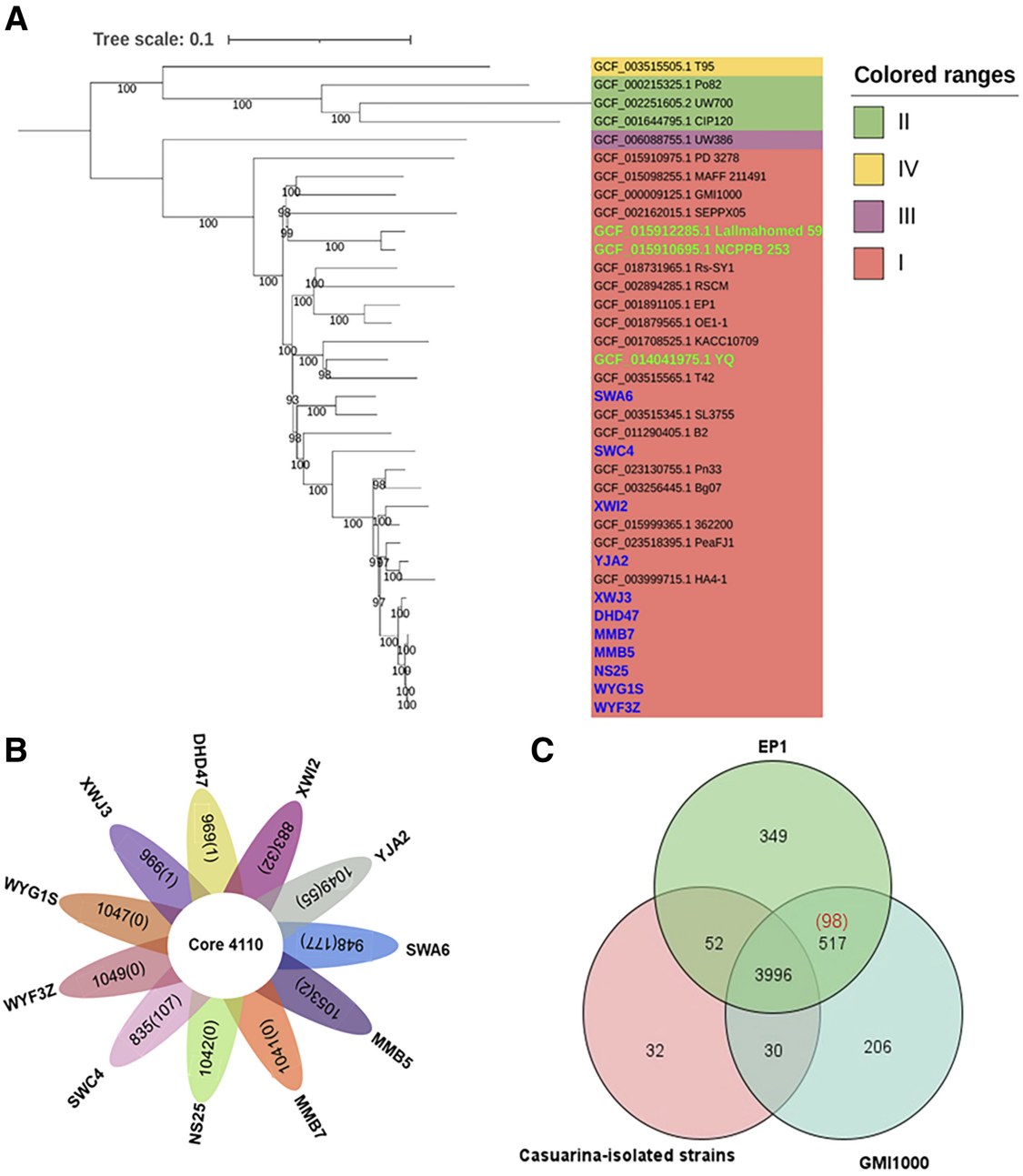

Fig. 2. Phylogenetic tree and comparative genomic analysis of *Casuarina*-isolated strains and other representative *Ralstonia solanacearum* species complex (RSSC) strains. **A,** Genome-based phylogenetic analysis classified the 11 *Casuarina*-isolated strains into RSSC phylotype I, forming a distinct lineage separate from three previously reported strains isolated from *Casuarina*, namely, NCPPB 253, Lallmahomed 59, and YQ. **B,** A core set of 4,110 genes are shared by all *Casuarina*-isolated strains, while each strain also contains up to 107 strain-specific genes (indicated by numbers in parentheses). The numbers outside parentheses indicate genes in each strain that are not part of the 4,110 core gene set. **C,** Venn diagram showing the genes specific to or shared by EP1, GMI1000, and all 11 *Casuarina*-isolated strains. A total of 98 genes (highlighted in red in parentheses) are shared by EP1 and GMI1000 but absent in all *Casuarina*-isolated strains.

with a premature stop codon in all *Casuarina*-isolated strains The *popC* gene encodes the avirulence effector RipAC of RSSC with a leucine-rich repeat sequence structural domain, which is secreted through the T3SS, and can prevent MAPK-mediated phosphorylation of SGT1 to suppress plant immunity (Yu et al. 2020) (Fig. 5C).

Type III secreted effectors (T3SEs) benefit the pathogens by suppressing plant defense mechanisms where these virulence factors

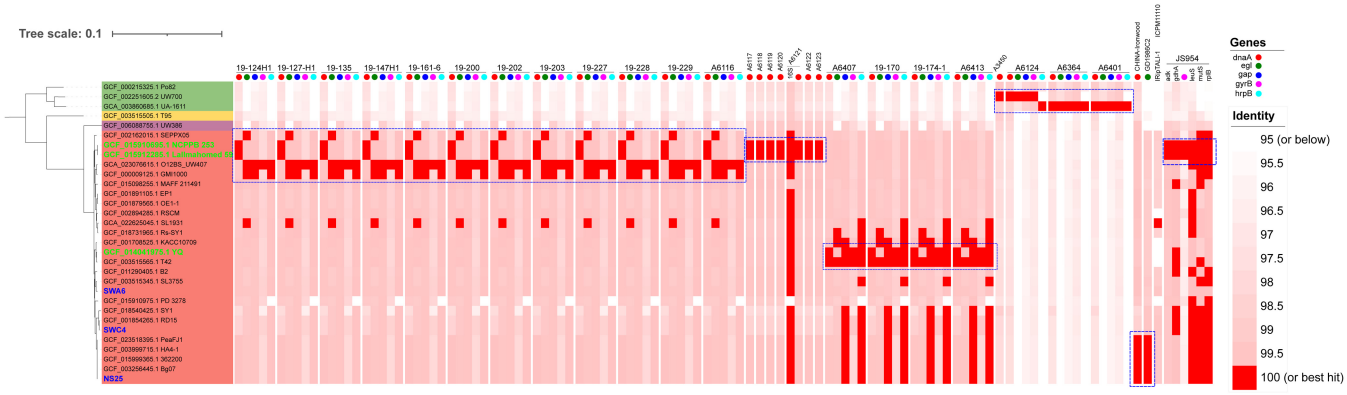

Fig. 3. Single-gene-based analysis of the diversity of *Casuarina*-associated *Ralstonia solanacearum* species complex (RSSC) strains. Single-gene nucleotide sequences available for *Casuarina*-associated RSSC strains were retrieved from the NCBI database and compared with whole-genome sequences of representative RSSC strains. Sequence similarity scores between single genes and their respective best match in genomes are shown as a heatmap. The phylogenetic tree on the left shows the evolutionary relationships between RSSC strains; three previously reported *Casuarina*-isolated strains (NCPPB 253, Lallmahomed 59, and YQ) are highlighted in green, while the three *Casuarina*-isolated strains collected in this study (SWA6, SWC4, and NS25) are highlighted in blue.

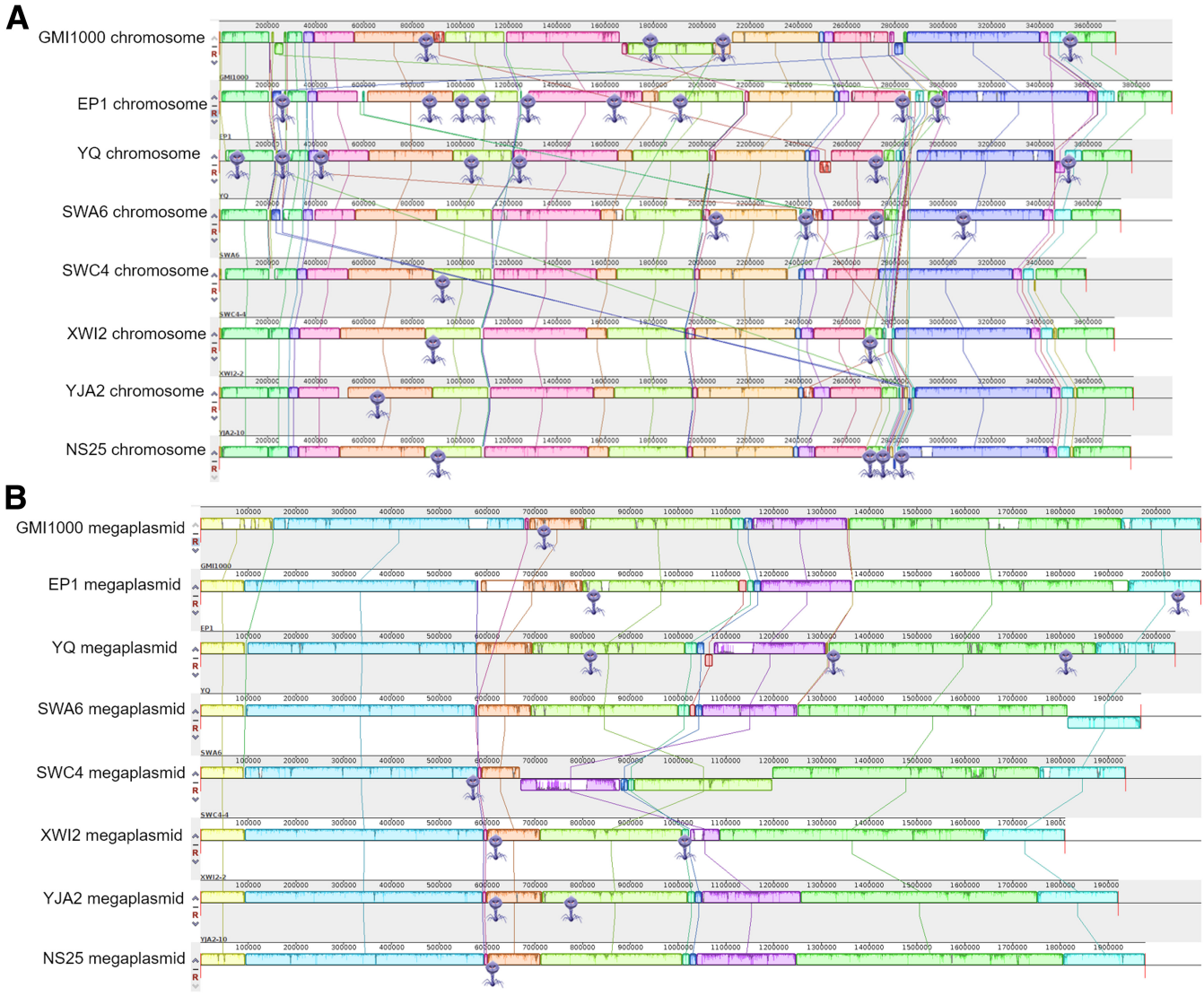

Fig. 4. Genome comparison of *Casuarina*-isolated strains and other representative *Ralstonia solanacearum* species complex strains. The results are shown separately for the chromosome (**A**) and megaplasmid (**B**). Prophages predicted in each genome are indicated by the "phage" icons on the chromosome/megaplasmid.

alter the physiology of the host cell. At the same time, certain effectors can be specifically recognized as avirulence factors by the host, leading to reduced pathogenicity (Ma et al. 2020). Approximately 70 T3SEs were predicted in the strains, 52 of which were shared by all strains (Supplementary Table S9). Two T3SEs (RipA5 and RipS8) were shared by EP1, GMI1000, and all *Casuarina*-isolated strains but absent in the peanut-isolated strain HA4-1. Four T3SEs (RipAY, RipG1, RipZ, and RipS3) are present in most strains except XWI2. Five T3SEs (RipAV, RipBM, RipS1, RipT, and RipP1) were shared by the strains except SWA6 and/or SWC4. Three T3SEs (RipAA, RipV1, and RipP2) were shared by GMI1000, EP1, SWA6, and/or SWC4. Importantly, five T3SEs (RipBO, RipAC, RipBJ, RS_T3E_Hyp6, and RipBP) were present in GMI1000 and/or EP1 but absent in all *Casuarina*-isolated strains, while four T3SEs (RipE2, RipAL, RipAX2, and RipS4) were shared by *Casuarina*-isolated strains but absent in GMI1000 and/or EP1. These T3SEs showing contrasting presence or absence patterns between GMI1000/EP1 and *Casuarina*-isolated strains might have contributed to the differential pathogenicity against *Casuarina* among strains (Fig. 5D).

Type IV secretion system: Type IV secretion system (T4SS) is involved in plasmid DNA transfer between bacteria, target localization, the proliferation of pathogens within the host cell, motility, secretion of virulence factors, and so on (Cabezón et al. 2015; Koo and Koh 2010). T4SS is encoded by a 14-kb-long gene cluster with 15 genes located on the chromosome. T4SS was only present in strains HA4-1, NS25, YJA2, and YQ, and the cluster organization was highly conserved in those strains (Supplementary Fig. S4B).

Type VI secretion system: Type VI secretion system (T6SS) is widespread in gram-negative bacteria. T6SS is a secretion system involved in pathogenesis, bacterial interactions, and competition. It can secrete antibacterial proteins into the target cells and thus kills the neighboring, nonimmune bacterial cells (Asolkar and Ramesh 2020). The T6SS gene cluster contains 16 core genes that are located in a 45-kb-long region on the megaplasmid. All of these core genes are present in all the RSSC strains examined here and are highly conserved in their protein sequences (>95% sequence identity, 100% coverage). At the same time, the middle region of the cluster was more variable among strains and mainly contained genes encoding IS5 transposon, MFS transporter, and putative proteins (Supplementary Fig. S4C).

Quorum sense system: Three quorum sensing (QS) systems have been discovered in phylotype I RSSC, namely, Phc, Sol, and Ras, which have critical roles in pathogenicity regulation (Li et al. 2016; Yan et al. 2022). Genes encoding each QS system are physically

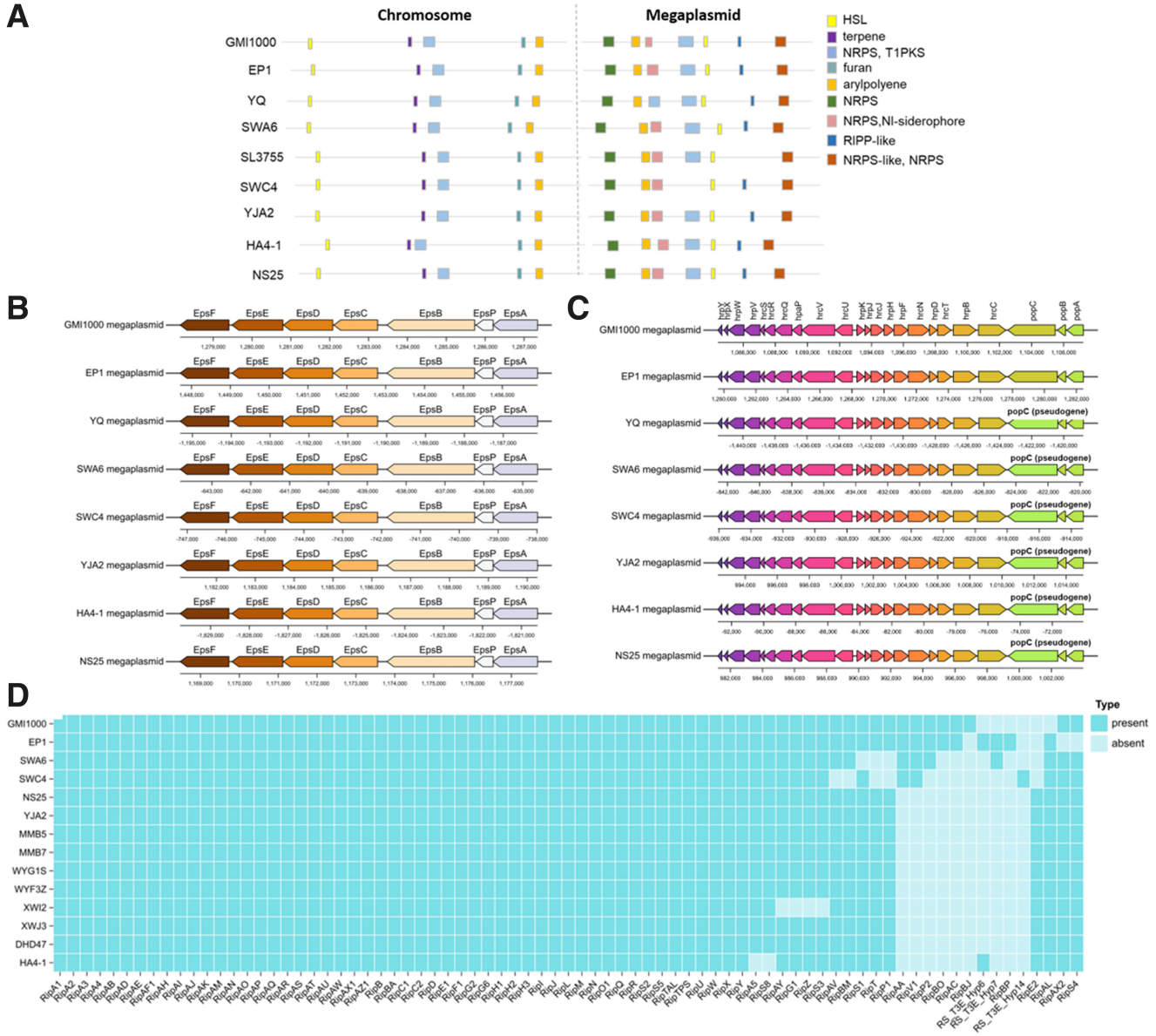

Fig. 5. Selected gene clusters of *Casuarina*-isolated strains and other representative *Ralstonia solanacearum* species complex strains. **A,** Secondary metabolite biosynthetic gene clusters. **B,** EPS biosynthetic gene cluster. **C,** Type III secretion system gene clusters. **D,** Repertoires of type III effector encoding genes.

linked in the genome, forming the *phcBSR*, *solIR*, and *rasIR* gene clusters, respectively. In addition, PhcA is a master regulator central to the pathogenicity regulatory network in RSSC and is activated in response to high cell density. The genes encoding *PhcA* and components of the three QS systems are present in all the eight representative RSSC strains from this study and are highly conserved in both their sequences (similarity >98% in all cases) and gene arrangements (Supplementary Fig. S5).

Discussion

RSSC is considered an excellent model for understanding the microevolutionary and macroevolutionary patterns leading to the emergence of ecotypes by adapting to new hosts and/or local environmental conditions. Since the sequencing of the first *R. solanacearum* genome was conducted in 2002 (Salanoubat et al. 2002), a total of 473 RSSC whole-genome sequences have been deposited in the NCBI genome database (as of 6 November 2023). This expansion in genomic resources has significantly facilitated the study of the pathogenic mechanisms of RSSC. Despite the initial discovery of *Casuarina* wilt in 1961 (Orian 1961), genome sequences are only available for three *Casuarina*-isolated RSSC strains, including NCPPB 253 and Lallmahomed 59 (from Africa) (Kurm et al. 2021) as well as YQ (from Zhejiang, China) (Zhou et al. 2021). Research on *Casuarina* wilt disease remained limited, as most studies of RSSC focused on strains isolated from herbaceous plants such as potatoes, tomatoes, and tobacco (Ailloud et al. 2015; Chen et al. 2022; Guarischi-Sousa et al. 2016; Sun et al. 2017), and fewer studies examined those from woody plants, let alone the comparisons between herbaceous and woody hosts.

In this study, we isolated 11 RSSC strains from the diseased *C. equisetifolia* at diverse locations along the coastline of Guangdong, China. These *Casuarina*-isolated strains, identified as RSSC phylotype I and biovar 4, shared close evolutionary relationships with each other and also with previously reported strains isolated from peanuts. Furthermore, they all showed high virulence against not only *C. equisetifolia* but also other herbaceous plants, including peanuts, tomatoes, and tobacco. Therefore, these RSSC strains likely represent causal agents of *Casuarina* wilt in Guangdong and might have driven the recent surge in disease cases. Their ability to infect plants other than *C. equisetifolia* might facilitate the long-term existence of these pathogens in the coastal area. The widespread distribution of these strains underscores a significant ecological challenge, suggesting the possibility of an extensive and sustained bacterial threat to the *Casuarina* coastal shelterbelts. This discovery emphasizes the necessity for ongoing surveillance and interventions in managing and mitigating the spread of this pathogen.

Given the successful eradication of *Casuarina* wilt using disease-resistant clones of *C. equisetifolia* in the 1980s, its recent re-emergence raises an intriguing question regarding the source of the pathogen. One possibility is that distinct RSSC strains caused the current and previous outbreaks. Alternatively, the original pathogens may have sustained and evolved to overcome the resistance of *Casuarina* trees after more than 3 decades of co-existence with the host. Our genomic and phylogenetic analyses of these strains shed light on their evolutionary trajectory within the RSSC. The current *Casuarina*-isolated strains represent a lineage different from NCPPB 253, Lallmahomed 59, and YQ in the genome-based phylogeny (Fig. 2A) (Kurm et al. 2021; Zhou et al. 2021). Importantly, as suggested by single-gene analysis, they potentially date back to strains existing in Guangdong since the 1980s (Fig. 3). This historical persistence and evolution within the region could have facilitated the emergence of the strains' current virulence characteristics.

The *Casuarina*-isolated strains exhibited considerably elevated virulence toward *C. equisetifolia* than GMI1000 and EP1, two representative RSSC strains initially isolated from tomatoes and eggplants. Our comparative genomic analysis between the *Casuarina*-isolated strains and these two representative strains uncovered unique genetic profiles. The presence or absence of specific genes in these strains could be pivotal in determining their virulence differences. Identifying and exploring these genes could unravel the molecular basis of their pathogenicity and offer avenues for targeted disease management. This genomic insight is key to understanding the mechanism underlying their differential pathogenicity.

EPS, secretion systems, and QS regulatory systems are known to have pivotal roles in the pathogenicity of plant pathogens. Microbial polysaccharides, long-chain polysaccharides formed by eukaryotes and prokaryotes during the growth and metabolism processes, are primarily responsible for cell adhesion and environmental protection. Polysaccharides are classified into three types based on their cellular locations: capsular polysaccharides, lipopolysaccharides, and EPSs (Yildiz and Karatas 2018). Major secretion systems of RSSC include T2SS, T3SS, T4SS, and T6SS, which secrete specific functional effector proteins to weaken host plants and facilitate infection (Abby et al. 2016; Asolkar and Ramesh 2020; Poueymiro and Genin 2009). Three QS systems have been discovered in RSSC strains, namely, Phc, Sol, and Ras, which have critical roles in pathogenicity regulation (Li et al. 2016; Yan et al. 2022). Our analyses showed that the gene clusters encoding the EPS I biosynthesis pathway, secretion systems, and QS systems are all highly conserved in the examined RSSC strains (Fig. 5B and C; Supplementary Figs. S4 and S5), with the only exception being the absence of the T4SS gene cluster in a few strains (EP1, SWA6, and SWC4) (Supplementary Fig. S4B). The results indicate that the RSSC strains from different hosts all share conserved sets of functional and regulatory machineries of pathogenicity.

Among secretion systems, T3SS is probably the most well-characterized one and has a major contribution to the pathogenicity of RSSC pathogens. It can inject type III effectors into plant cells to interfere with various host cellular mechanisms, including suppression of the innate immune system, alteration of cell signaling pathways, and hijacking of the metabolic processes of the hosts to facilitate the infection of pathogens (Hueck 1998; Pensec et al. 2015). Approximately 90 type III effector protein families have been identified in the RSSC pangenome, with each strain containing about 70 T3SEs (Peeters et al. 2013). Many of these effectors have been identified as virulence factors contributing to bacterial fitness during infection (Deslandes and Genin 2014). For instance, RipI can hijack plant metabolism to support pathogen nutrition (Xian et al. 2020). RipAB can target TGA transcription factors to subvert salicylic acid signaling (Qi et al. 2022). On the contrary, the presence of certain effectors can elicit the immune response on some hosts and thus limit the host range of the pathogen. In *R. pseudosolanacearum* GMI1000, AvrA (RipAA) and PopP1 (RipP1) are found to be major host specificity determinants recognized by *Nicotiana tabacum*/*N. benthamiana* and *N. glutinosa*, respectively (Poueymiro and Genin 2009).

Comparative analysis of the *Casuarina* isolates, GMI1000, and EP1 detected 77 families of type III effectors. Most of these effectors were shared by the strains used in this study (Fig. 5D), suggesting a conserved core set of effectors important for the virulence of these pathogens on their common hosts, such as tomato and tobacco. On the other hand, nine effectors exhibited presence or absence patterns that are consistent with the differential virulence of strains against *C. equisetifolia* and peanut (Fig. 5D). Four of these effectors (RipE2, RipAL, RipS4, and RipAX2) were present in nearly all *Casuarina* and peanut isolated strains (high virulence) but absent in GMI1000 and/or EP1 (low virulence), suggesting that they may positively contribute to the pathogens' virulence on *C. equisetifolia* and peanut. Among them, RipE2 has been reported to be absent in phylotype I strains (Peeters et al. 2013), raising the possibility of its acquisition in *Casuarina*-isolated strains through horizontal gene transfer. RipAL and RipAX2 were found to induce the defense responses against the RSSC (Morel et al. 2018; Nakano and Mukaihara 2019), while RipS4 was important for bacterial fitness in eggplant (K. Chen et al. 2021). In contrast, five other effectors (RipBO, RipAC, RipBJ, RS_T3E_Hyp6, and RipBP) were present in GMI1000 and/or EP1 but absent in all the *Casuarina*-isolated strains, suggesting that their presence might compromise the pathogens' virulence on *C. equisetifolia*. RipBJ can interact with the plant NADPH oxidase

SlWfi1 and lead to reduced virulence in plants (Chu 2021). A recent study showed that RipAC can suppress effector-triggered immunity in tobacco (Yu et al. 2020). However, the effectors RipBO, RS_T3E_Hyp6, and RipBP have not been functionally characterized. These nine effectors might be promising candidates for further functional investigations to understand the pathogenic mechanisms of RSSC strains on *C. equisetifolia*.

By isolating and characterizing the RSSC strains underlying the recent outbreak of Casuarina bacterial wilt in Guangdong, elucidating their phylogenetic diversity, as well as identifying genomic differences between the Casuarina-isolated RSSC strains and those from other hosts, this research offers a critical foundation for developing targeted interventions. The discovery of unique genes in the Casuarina-isolated strains that are absent in other RSSC reference strains (GMI1000 and EP1) is particularly promising. These unique genetic markers can lead to precise diagnostic assays for early detection of the pathogen, enabling timely implementation of control measures. Furthermore, understanding host-specific adaptations that contribute to higher virulence in *C. equisetifolia* could open avenues for breeding disease-resistant Casuarina varieties by focusing on these specific genetic interactions.

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Literature Cited

Abby, S. S., Cury, J., Guglielmini, J., Néron, B., Touchon, M., and Rocha, E. P. C. 2016. Identification of protein secretion systems in bacterial genomes. Sci. Rep. 6:23080.

Ailloud, F., Lowe, T., Cellier, G., Roche, D., Allen, C., and Prior, P. 2015. Comparative genomic analysis of *Ralstonia solanacearum* reveals candidate genes for host specificity. BMC Genomics 16:270.

Aoun, N., Georgoulis, S. J., Avalos, J. K., Grulla, K. J., Miqueo, K., Tom, C., and Lowe-Power, T. M. 2023. A pangenomic atlas reveals that eco-evolutionary dynamics shape plant pathogen type VI secretion systems. BioRxiv https://doi.org/10.1101/2023.09.05.556054

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., and Wishart, D. S. 2016. PHASTER: A better, faster version of the PHAST phage search tool. Nucleic Acids Res. 44:W16-W21.

Asolkar, T., and Ramesh, R. 2020. The involvement of the Type Six Secretion System (T6SS) in the virulence of *Ralstonia solanacearum* on brinjal. 3 Biotech 10:324.

Ayin, C. M., Alvarez, A. M., Awana, C., Schleinzer, F. M., Marx, B. D., and Schlub, R. L. 2019. *Ralstonia solanacearum*, *Ganoderma australe*, and bacterial wetwood as predictors of ironwood tree (*Casuarina equisetifolia*) decline in Guam. Australas. Plant Pathol. 48:625-636.

Ayin, C. M., Schlub, R. L., Yasuhara-Bell, J., and Alvarez, A. M. 2015. Identification and characterization of bacteria associated with decline of ironwood (*Casuarina equisetifolia*) in Guam. Australas. Plant Pathol. 44:225-234.

Balamurugan, A., Muthamilan, M., Kamalakannan, A., Shanthi, A., and Arumugam, T. 2020. Characterization of *Ralstonia solanacearum* causing bacterial wilt disease of tomato in Coimbatore District of Tamil Nadu, India. Int. J. Curr. Microbiol. Appl. Sci. 9:3010-3016.

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S. Y., Medema, M. H., and Weber, T. 2019. antiSMASH 5.0: Updates to the secondary metabolite genome mining pipeline. Nucleic Acids Res. 47:W81-W87.

Buddenhagen, I., and Kelman, A. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 2:203-230.

Cabezón, E., Ripoll-Rozada, J., Peña, A., de la Cruz, F., and Arechaga, I. 2015. Towards an integrated model of bacterial conjugation. FEMS Microbiol. Rev. 39:81-95.

Cellier, G., Pecrix, Y., Gauche, M. M., and Cheron, J. J. 2023. *Ralstonia solanacearum* species complex *egl* reference database. CIRAD Dataverse https://doi.org/10.18167/DVN1/CUWA5P

Chen, K., Wang, L., Chen, H., Zhang, C., Wang, S., Chu, P., Li, S., Fu, H., Sun, T., Liu, M., Yang, Q., Zou, H., and Zhuang, W. 2021. Complete genome sequence analysis of the peanut pathogen *Ralstonia solanacearum* strain Rs-P.362200. BMC Microbiol. 21:118.

Chen, K., Zhuang, Y., Wang, L., Li, H., Lei, T., Li, M., Gao, M., Wei, J., Dang, H., Raza, A., Yang, Q., Sharif, Y., Yang, H., Zhang, C., Zou, H., and Zhuang, W. 2022. Comprehensive genome sequence analysis of the devastating tobacco bacterial phytopathogen *Ralstonia solanacearum* strain FJ1003. Front. Genet. 13:966092.

Chen, T., Zhang, H., Liu, Y., Liu, Y.-X., and Huang, L. 2021. EVenn: Easy to create repeatable and editable Venn diagrams and Venn networks online. J. Genet. Genom. 48:863-866.

Chu, L.-W. 2021. Identification and Characterization of Plant Proteins Interacting with *Ralstonia solanacearum* Effector RipBJ. National Taiwan University, Taipei, Taiwan. https://hdl.handle.net/11296/g76bn5

da Silva Xavier, A., de Melo, A. G., Hendrich, C. G., Tremblay, D. M., Rousseau, G. M., Plante, P.-L., Forest, K. T., Alfenas-Zerbini, P., Allen, C., and Moineau, S. 2022. In through the out door: A functional virulence factor secretion system is necessary for phage infection in *Ralstonia solanacearum*. mBio 13: e0147522.

Darling, A. E., Mau, B., and Perna, N. T. 2010. progressiveMauve: Multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5:e11147.

Deslandes, L., and Genin, S. 2014. Opening the *Ralstonia solanacearum* type III effector tool box: Insights into host cell subversion mechanisms. Curr. Opin. Plant Biol. 20:110-117.

Emms, D. M., and Kelly, S. 2019. OrthoFinder: Phylogenetic orthology inference for comparative genomics. Genome Biol. 20:238.

Fegan, M., and Prior, P. 2005. How complex is the *Ralstonia solanacearum* species complex? Pages 449-461 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Genin, S., and Boucher, C. 2004. Lessons learned from the genome analysis of *Ralstonia solanacearum*. Annu. Rev. Phytopathol. 42:107-134.

Gosselin, S., Fullmer, M. S., Feng, Y., and Gogarten, J. P. 2022. Improving phylogenies based on average nucleotide identity, incorporating saturation correction and nonparametric bootstrap support. Syst. Biol. 71:396-409.

Guarischi-Sousa, R., Puigvert, M., Coll, N. S., Siri, M. I., Pianzzola, M. J., Valls, M., and Setubal, J. C. 2016. Complete genome sequence of the potato pathogen *Ralstonia solanacearum* UY031. Stand. Genomic Sci. 11:7.

Guo, Q., and Liang, Z. 1986. Screening techniques and integrated control measures for *Ralstonia solanacearum* resistant strains of *Casuarina equisetifolia*. Pract. For. Technol. 4:7-9.

Huang, J., and Schell, M. 1995. Molecular characterization of the *eps* gene cluster of *Pseudomonas solanacearum* and its transcriptional regulation at a single promoter. Mol. Microbiol. 16:977-989.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62:379-433.

Ji, X., Tang, J., and Zhang, J. 2022. Effects of salt stress on the morphology, growth and physiological parameters of *Juglansmicrocarpa* L. seedlings. Plants 11:2381.

Jiang, G., Wei, Z., Xu, J., Chen, H., Zhang, Y., She, X., Macho, A. P., Ding, W., and Liao, B. 2017. Bacterial wilt in China: History, current status, and future perspectives. Front. Plant Sci. 8:1549.

Koo, J.-E., and Koh, Y.-S. 2010. Structural and transcriptional analysis of gene clusters for a type IV secretion system in *Orientia tsutsugamushi*. World J. Microbiol. Biotechnol. 26:753-759.

Kurm, V., Houwers, I., Coipan, C. E., Bonants, P., Waalwijk, C., van der Lee, T., Brankovics, B., and van der Wolf, J. 2021. Whole genome characterization of strains belonging to the *Ralstonia solanacearum* species complex and in silico analysis of TaqMan assays for detection in this heterogenous species complex. Eur. J. Plant Pathol. 159:593-613.

Li, P., Wang, D., Yan, J., Zhou, J., Deng, Y., Jiang, Z., Cao, B., He, Z., and Zhang, L. 2016. Genomic analysis of phylotype I strain EP1 reveals substantial divergence from other strains in the *Ralstonia solanacearum* species complex. Front. Microbiol. 7:1719.

Liu, Y., Wu, D., Liu, Q., Zhang, S., Tang, Y., Jiang, G., Li, S., and Ding, W. 2016. The sequevar distribution of *Ralstonia solanacearum* in tobacco-growing zones of China is structured by elevation. Eur. J. Plant Pathol. 147:541-551.

Liang, Z. C., and Chen, X. H. 1984. Selection of clones of *Carsuarina* for resistances to bacterial wilt. J. South China Agric. Coll. 5:53-59.

Ma, W., Xu, X., Cai, L., Cao, Y., Haq, F., Alfano, J. R., Zhu, B., Zou, L., and Chen, G. 2020. A *Xanthomonas oryzae* type III effector XopL causes cell death through mediating ferredoxin degradation in *Nicotiana benthamiana*. Phytopathol. Res. 2:16.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S. V., Machado, M. A., Toth, I., Salmond, G., and Foster, G. D. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Mol. Plant Pathol. 13:614-629.

Meng, F. 2013. *Ralstonia solanacearum* species complex and bacterial wilt disease. Meng. J. Bacteriol. Parasitol. 4:e119.

Morel, A., Guinard, J., Lonjon, F., Sujeeun, L., Barberis, P., Genin, S., Vailleau, F., Daunay, M.-C., Dintinger, J., Poussier, S., Peeters, N., and Wicker, E. 2018. The eggplant AG91-25 recognizes the Type III-secreted effector RipAX2 to trigger resistance to bacterial wilt (*Ralstonia solanacearum* species complex). Mol. Plant Pathol. 19:2459-2472.

Nakano, M., and Mukaihara, T. 2019. Comprehensive identification of PTI suppressors in type III effector repertoire reveals that *Ralstonia solanacearum* activates jasmonate signaling at two different steps. Int. J. Mol. Sci. 20:5992.

Peeters, N., Carrère, S., Anisimova, M., Plener, L., Cazalé, A.-C., and Genin, S. 2013. Repertoire, unified nomenclature and evolution of the Type III effector gene set in the *Ralstonia solanacearum* species complex. BMC Genomics 14:859.

Peng, G. 2000. Growth comparison of *Pseudomonas solanacearum* resistance *Casuarina equisetifolia*. Guangdong For. Sci. Technol. 16:35-37.

Pensec, F., Lebeau, A., Daunay, M. C., Chiroleu, F., Guidot, A., and Wicker, E. 2015. Towards the identification of type III effectors associated with

Ralstonia solanacearum virulence on tomato and eggplant. Phytopathology 105:1529-1544.

Potgieter, L. J., Richardson, D. M., and Wilson, J. R. U. 2014a. *Casuarina cunninghamiana* in the Western Cape, South Africa: Determinants of naturalisation and invasion, and options for management. S. Afr. J. Bot. 92:134-146.

Potgieter, L. J., Richardson, D. M., and Wilson, J. R. U. 2014b. *Casuarina*: Biogeography and ecology of an important tree genus in a changing world. Biol. Invasions 16:609-633.

Poueymiro, M., and Genin, S. 2009. Secreted proteins from *Ralstonia solanacearum*: A hundred tricks to kill a plant. Curr. Opin. Microbiol. 12:44-52.

Poussier, S., Chéron, J.-J., Couteau, A., and Luisetti, J. 2002. Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates. J. Microbiol. Methods 51:349-359.

Prior, P., Ailloud, F., Dalsing, B. L., Remenant, B., Sanchez, B., and Allen, C. 2016. Genomic and proteomic evidence supporting the division of the plant pathogen *Ralstonia solanacearum* into three species. BMC Genomics 17:90.

Qi, P., Huang, M., Hu, X., Zhang, Y., Wang, Y., Li, P., Chen, S., Zhang, D., Cao, S., Zhu, W., Xie, J., Cheng, J., Fu, Y., Jiang, D., Yu, X., and Li, B. 2022. A *Ralstonia solanacearum* effector targets TGA transcription factors to subvert salicylic acid signaling. Plant Cell 34:1666-1683.

Safni, I., Cleenwerck, I., De Vos, P., Fegan, M., Sly, L., and Kappler, U. 2014. Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: Proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. Int. J. Syst. Evol. Microbiol. 64:3087-3103.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thébault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J., and Boucher, C. A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

Sun, S., Shu, C. W., Chen, J. L., Wang, J., and Zhou, E. X. 2014. Screening for resistant clones of *Casuarina equisetifolia* to bacterial wilt and the analysis of AFLP markers in resistant clones. For. Pathol. 44:276-281.

Sun, Y., Wang, K., Caceres-Moreno, C., Jia, W., Chen, A., Zhang, H., Liu, R., and Macho, A. P. 2017. Genome sequencing and analysis of *Ralstonia solanacearum* phylotype I strains FJAT-91, FJAT-452 and FJAT-462 isolated from tomato, eggplant, and chili pepper in China. Stand. Genomic Sci. 12:29.

Xian, L., Yu, G., Wei, Y., Rufian, J. S., Li, Y., Zhuang, H., Xue, H., Morcillo, R. J. L., and Macho, A. P. 2020. A bacterial effector protein hijacks plant metabolism to support pathogen nutrition. Cell Host Microbe 28:548-557.e7.

Xu, J., Pan, Z. C., Prior, P., Xu, J. S., Zhang, Z., Zhang, H., Zhang, L. Q., He, L. Y., and Feng, J. 2009. Genetic diversity of *Ralstonia solanacearum* strains from China. Eur. J. Plant Pathol. 125:641-653.

Yan, J., Li, P., Wang, X., Zhu, M., Shi, H., Yu, G., Chen, X., Wang, H., Zhou, X., Liao, L., and Zhang, L. 2022. RasI/R quorum sensing system controls the virulence of *Ralstonia solanacearum* strain EP1. Appl. Environ. Microbiol. 88:e0032522.

Yildiz, H., and Karatas, N. 2018. Microbial exopolysaccharides: Resources and bioactive properties. Process Biochem. 72:41-46.

Yu, G., Xian, L., Xue, H., Yu, W., Rufian, J. S., Sang, Y., Morcillo, R. J. L., Wang, Y., and Macho, A. P. 2020. A bacterial effector protein prevents MAPK-mediated phosphorylation of SGT1 to suppress plant immunity. PLoS Pathog. 16:e1008933.

Zhang, Y., Hu, P., Zhong, C., Wei, Y., Meng, J., Li, Z., Pinyopusarerk, K., and Bush, D. 2020. Analyses of genetic diversity, differentiation and geographic origin of natural provenances and land races of *Casuarina equisetifolia* based on EST-SSR markers. Forests 11:432.

Zhong, C., Zhang, Y., Chen, Y., Jiang, Q., Chen, Z., Liang, J., Pinyopusarerk, K., Franche, C., and Bogusz, D. 2010. *Casuarina* research and applications in China. Symbiosis 50:107-114.

Zhong, C., Zhang, Y., Wei, Y., Meng, J., Chen, Y., Bush, D., Bogusz, D., and Franche, C. 2019. The role of *Frankia* inoculation in *Casuarina* plantations in China. Antonie van Leeuwenhoek 112:47-56.

Zhou, X., Wang, Y., Li, C., Xu, Y., Su, X., Yang, T., and Zhang, X. 2021. Differential expression pattern of pathogenicity-related genes of *Ralstonia pseudosolanacearum* YQ responding to tissue debris of *Casuarina equisetifolia*. Phytopathology 111: 1918-1926.