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ORIGINAL RESEARCH ARTICLE

The genomes of two Australian isolates of *Verticillium dahliae* recovered from cotton fields

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Abstract

Verticillium wilt is a major disease in a wide variety of crops and is caused by the fungus *Verticillium dahliae Kleb.* In Australian cotton growing regions two pathotypes of *V. dahliae* are described, namely non-defoliating and defoliating, classified on their ability to cause defoliation in cotton (*Gossypium hirsutum*) as well as okra (*Abelmoschus esculentus)* and olives (*Olea europaea*). Herein we report the genomes of two isolates of *V. dahliae*, one predicted to be non-defoliating and the other predicted to be defoliating. Phylogenomic analysis places each isolate into separate clades, but the highly aggressive, predicted defoliating, strain lacks the genomic features reported as important for causing defoliation on cotton in other regions.

Keywords Verticllium wilt · Cotton · Genome · Vegetative compatibility group

Introduction

Verticillium dahliae is a fungus from the Sordariomycete class within the Ascomycota. It is a soil-borne pathogen of hundreds of plants including many important food and fibre crops such as cotton, olives, tomatoes and potatoes (Chen et al. 2021). In most plants it behaves as a hemibiotroph, initially invading intercellularly in the root cortex before entering the xylem vessels of the plant where it can proliferate and sporulate and ultimately block the transpiration steam resulting in wilting and ultimately death of the plant. The microsclerotia that form following infection can survive in the soil for more than a decade (Dadd-Daigle et al. 2021; Chen et al. 2021). Together with the lack of suitable fungicides to access the below ground tissue or strong immune-type resistance in most crops, the persistence of the fungus in the soil severely limits management options.

Despite the presence of two alternate mating-types in V. dahliae populations, previous studies have shown largely clonal populations structures with some limited, possibly historical, recombination (Milgroom et al. 2014). There are various classification systems for differentiation of strains of *V. dahliae* including the use of vegetative compatibility groupings (VCGs), race designations with respect to virulence/avirulence interactions on tomato differentials, phenotypic classifications based on a strain's ability to defoliate crops such cotton, as well as lineage discrimination based on molecular markers. While there is considerable overlap between these classification systems, for example the defoliating strains of *V. dahliae* are exclusively found in VCG1A/B, there are other complexities to these systems. For example the VCGs in many cases are subdivided based upon the vigour of the interactions in compatibility testing (Joaquim and Rowe 1991). These subgroupings are largely supported by molecular markers suggesting a polyphyletic nature of the major VCGs. For example, VCG2 is divided into VCG2A, VCG2B^{234}, VCG2B^{824} and possibly one additional clade (Milgroom et al. 2014; Collado-Romero et al. 2008). VCG4 is also split into two groups, VCG4A and VCG4B (Milgroom et al. 2014; Collado-Romero et al. 2008). The VCG classification appears further complicated by some recombinant lineages (Milgroom et al. 2014). The defoliating/non-defoliating classification is also nuanced. Indeed the non-defoliating strains can cause disease as

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severe as that caused by defoliating strains and there appears to be a gradient of aggressiveness of isolates despite the binary defoliating/non-defoliating classification system (Korolev et al. 2001). Moreover some authors make use of a defoliating-like designation in addition to the defoliating and non-defoliating designations (Korolev et al. 2008). Cotton-derived isolates from Australia also do not appear to always conform to the defoliating/non-defoliating pathotype classification, with many VCG2A isolates (which are typically considered non-defoliating) able to cause severe disease and defoliate plants (Webster et al. 2023; Dadd-Daigle et al. 2020).

Verticillium dahliae was originally reported in Australian cotton growing regions as far back as 1959 (Kirkby et al. 2013). Prior to the 2013/2014 cropping season, it was thought there was only a single VCG type present in Australian cotton fields; however, this changed with the detection of the non-defoliating VCG2A strain in 2014 (Smith et al. 2014) and the defoliating strain in 2016 (Chapman et al. 2016). It is a significant problem for cotton production throughout the cotton growing regions (Dadd-Daigle et al. 2021).

The complexities with the classification systems, coupled with the extremely broad host range of *V. dahliae*, and at times anecdotal observations of strain phenotypes, means we need a greater understanding of the global variation in *V. dahliae* and how this relates to field diseases. Whole genome sequencing is now within reach of many laboratories globally and will likely replace other molecular marker systems with the advantage of the data, if made publicly available, being reusable. Towards this end we here report the genomes of two Australian isolates of *V. dahliae* and position these within a set of global isolates of *V. dahliae* which hopefully in the future can be expanded to fully understand the population structure of this important pathogen.

Materials and methods

Isolation

Cotton plants suspected of being infected with the Verticillium wilt pathogen, *V. dahliae*, based on foliar and internal disease symptoms were collected from commercial cotton fields in NSW in the 2017/18 season. For recovery of the pathogen, a 10 cm section of stem cut from the base of the plant was washed under running tap water, surface sterilised in 2% sodium hypochlorite for 2 min, rinsed three times in sterile distilled water, then airdried on sterilized paper towel. Using aseptic technique, the bark was peeled back, and small pieces of discoloured vascular tissue were removed and plated onto quarter strength potato dextrose agar growth medium amended with 100 mg/L of streptomycin (¼PDA/S). Plates were incubated at room temperature in the laboratory for 7 to 10 days. Three representative isolates per stem identified as *V. dahliae* based on morphological and cultural characteristics, including the production of microsclerotia and conidia produced on verticillately branched conidiophores, were sub-cultured from isolation plates and single spored. To single spore, 100–500 spores were transferred onto a plate of ¼PDA/S using a sterile loop and spread on the surface of the medium by a glass spreader using 0.1 mL of sterile distilled water. The inoculated media was incubated at room temperature in the laboratory for 16–18 h. Plates were examined under a stereo light microscope for germination of spores. Two germinated spores were selected and transferred to new ¼PDA/S growth plates, grown for 7 days at room temperature then stored under water at 4 °C. Isolates were deposited in the Queensland Plant Pathology Herbarium collection (Ecosciences Precinct, Dutton Park, Qld) and used in this study (Table 1). BRIP 71172 was isolated from the MacIntyre Valley in New South Wales Australia and isolate BRIP 71181 was isolated from the Gwydir Valley, New South Wales, Australia.

A pathology assay with isolate BRIP 71181 was conducted in a controlled environment facility maintained at

Table 1 Genome assembly metrics and meta data for two Australian isolates of Verticillium dahliae from cotton fields

Isolate	Predicted pathotype	Coverage (fold)	Contigs	Scaffolds	Busco statistics complete duplicated fragmented missing	Percentage of assembly assigned to chromosomes	Assembly length (Mbp)	Locus tag	Accession
BRIP 71181	Defoliating	30	2,589	163	C:93.1% D:0.1% F:1.6% M:5.3%	98.4	31.7	VDGD	QEPA01000000
BRIP 71172	Non-defoliating	1100	805	277	C:95.4% D:0.2%] F:1.1% M:3.5%	76	33.3	VDGE	RSDZ01000000

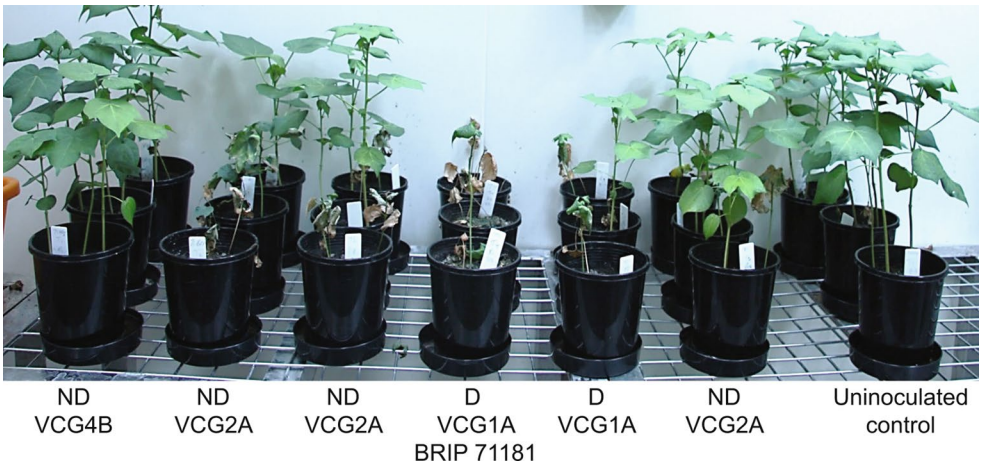

Fig. 1 Pathogenicity of various field isolates of Verticillium dahliae toward cotton cv Sicot 71BRF. Isolate BRIP 71181 is labelled with its presumed pathoytpe (D, defoliating) and VCG based on a PCR assay and phylogenetic analyses (see later). Other isolates in this experiment do not have accessions but had their VCGs and pathotype designated via PCR (data not shown)

26 °C with 65% relative humidity and a 12/12 hour day/night cycle. Cotton cultivar Sicot 71BRF plants were root dip inoculated into a spore suspension at 1×10^6 spores mL^{-1} as described elsewhere (Chen et al. 2024). The putative pathotype of BRIP 71181 was confirmed using a PCR assay (Supplementary Fig. 1) as previously described (Mercado-Blanco et al. 2003).

DNA extraction, DNA sequencing, genome assembly and annotation

Conidia ($\sim 1 \times 10^6$) were used to inoculate 20 mL of half strength potato dextrose broth in a deep 9 cm Petri dish and allowed to grow for 5 days at room temperature in the dark without agitation. Mycelia were harvested by filtration through Miracloth (Millipore), freeze dried, ground to a fine powder using a Retsch Mixer mill in 2 mL Eppendorf tubes with a single ball bearing and subjected to DNA extraction using QIAgen DNeasy Plant mini-spin gDNA extraction kit according to the manufacturer's instructions.

Illumina gDNA libraries were prepared and sequenced by the Australian Genome Research Facility (Melbourne, Australia) on either a HiSeq2500 (isolate BRIP 71181, 125 bp paired end reads) or NovoSeq6000 (isolate BRIP 71172, 150 bp paired end reads). Reads were quality trimmed and assembled as previously described (Gardiner 2018). Contigs were uploaded to NCBI where they were screened for contaminants by the NCBI pipelines, with contaminants removed prior to any further analysis. Contigs were further scaffolded to chromosomes via alignment to the VdLs.17 genome (Klosterman et al. 2011) using the PROmer, part of the MUMmer genome aligner (Marçais et al. 2018), with the output parsed into a golden paths file (AGP) for upload to NCBI to allow scaffolding in the final genome release. Protein coding genes were annotated as previously described (Gardiner 2018) with BRAKER v2.1.0 using RNAseq data from other samples of *V. dahliae* downloaded from NCBI to train the predictor. Reciprocal best blast hits were used between BRIP 71181 and the protein from VdLs.17 to maintain, where possible, matching numbers in the locus tags identifiers. Genes that did not have reciprocal best BLAST hits (RBBHs) with the VdLs.17 protein were given locus tag numbers starting at 20,001 in isolate BRIP 71181. Subsequent RBBHs were used between isolate BRIP 71181 and BRIP 71172 to again maintain the locus tag numbers. Genes in BRIP 71172 that did not have clear RBBHs with isolate BRIP 71181 were given numbers starting 30,001.

BUSCO analysis

BUSCO (5.5.0) (Manni et al. 2021) was run in the Galaxy computing environment. Default options were used with the exception of the selection the lineage as the Ascomycota. As a reference dataset the telomere-telomere assembly of *V. dahliae* VdLs.17 genome (GenBank Accession GCA_000952015.1) was used.

Phylogenomic analysis

Raw reads of 24 isolates of *V. dahliae* (including the two isolates sequenced here) plus *Verticillium alfalfae* were downloaded from NCBI into the Galaxy environment. Reads were adaptor and quality trimmed using Trimmomatic (v0.36.6) (Bolger et al. 2014) including the Illumina clip with TruSeq3 adaptors, filtering for a contiguous region of a minimum of a Phred quality of 29 and discarding of sequences below 50 base pairs. Pairs of reads that passed these filters were retained. Reads were aligned to the VdLs.17 genome (GenBank accession GCA_000150675.2) using Bowtie2 (v2.5.0) (Langmead and Salzberg 2012) run with default parameters. Bam alignment file headers were edited using AddOrReplaceReadGroups in the Galaxy environment. FreeBayes (v1.3.6) (Garrison and Marth 2012) run with minimum coverage of three and the population model

set to haploid was used to call variant loci and output a single vcf file (Supplementary data 1). The read files downloaded from NCBI can be identified by the sequence read archive accessions in the vcf file.

The dataset of Milgroom et al. (2014) was incorporated as follows. The hapmap file from the supplementary file of that publication was converted to a vcf file using TASSEL (v5.2.92) (Bradbury et al. 2007). The vcf file was corrected based on the reference sequence using bcftools norm (v1.15.1) and the ploidy set to 1 for all loci using bcftools fixploidy (Li et al. 2009) in the Galaxy environment. The FreeBayes output was filtered to include only loci identified by Milgroom prior to joining the Milgroom and Freebayes calls using bcftools merge. Loci that had calls in a minimum of 151 isolates were retained using the bcftools filter function with AN > 150. This left a total of 17,238 loci.

The final vcf file consisting of SNP data for 166 isolates (supplementary data 1) was passed through VCF-kit (Cook and Andersen 2017) ('vk phylo fasta' command run with default parameters) to extract a fasta alignment of all the 17,238 loci. About half the isolates using the alignment were removed at this stage to reduce the final size of the phylogenetic tree as there was a substantial number of isolates in the Milgroom et al. (2014), particularly in the VCG1A/B lineage. RAxML gui 2.0 (Edler et al. 2021) was used to build a phylogenetic tree with the following options; binary was raxml-ng, substitution model GTR, ascertainment bias Lewis' method, with maximum likelihood analysis with a transfer to bootstrap expectation and consensus with a single run with 100 replicates and the outgroup set as *Verticillium alfalfae* (supplementary file 2). The tree was viewed in iTOL (Letunic and Bork 2021) for export to Adobe Illustrator for final figure annotation.

Analysis of the defoliating lineage specific region

Reads for the defoliating strains Vd991 and VDG47 (Zhang et al. 2019) and the non-defoliating strains JR2, VdLs.16 and VDG69, along with the two isolates sequenced here were aligned to the chromosomal assembly of Vd991 (Yang et al. 2023). Alignment of reads were performed as described above. Coverage graphs were generated using bamCoverage (v3.5.4) (Ramírez et al. 2016) in the Galaxy environment. In the absence of a publicly available full gene annotation for the genome of Vd991, the sequences for the defoliating region genes available in genbank accession MF946582 were used to identify the region in the larger assembly using BLAT (Kent 2002). The gene and read coverage graphs were visualised in the JBrowse2 software (v2.10.1) (Diesh et al. 2023).

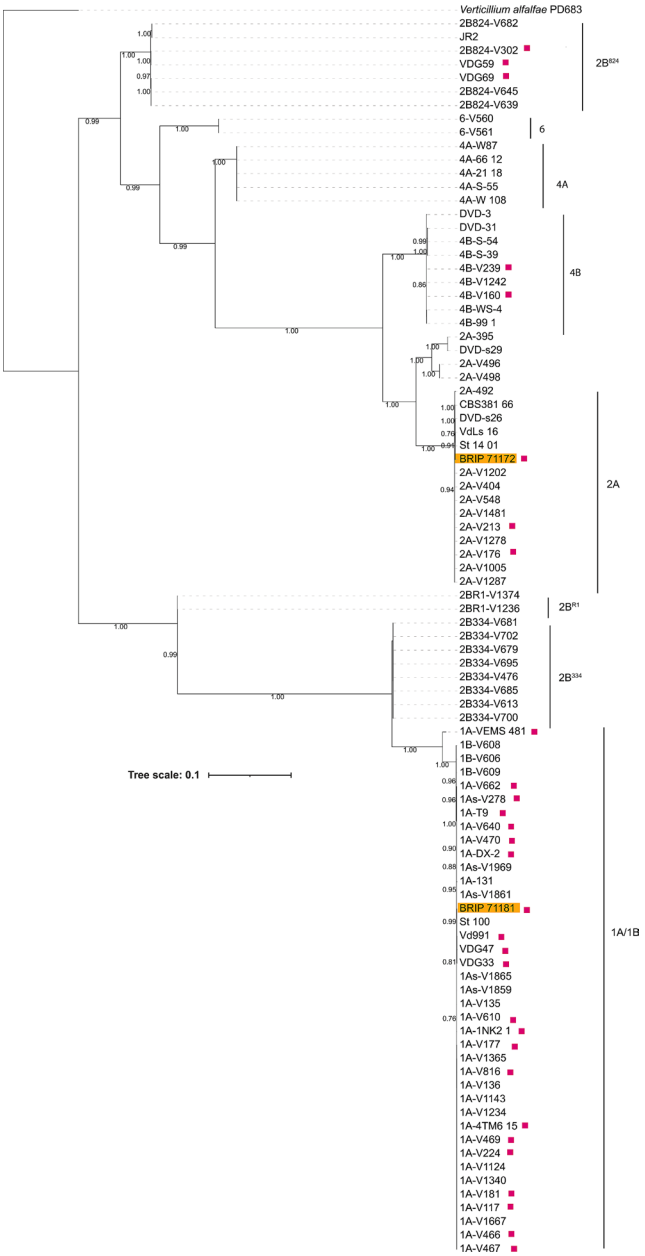

Fig. 2 Genome wide phylogeny of a global set of Verticillium dahliae isolates positions the two Australian isolates in different Verticillium dahliae clades; 2 A (BRIP 71172) and 1 A/1B (BRIP 71181). Isolates originally recovered from cotton plants are marked with a pink box with these isolates either described in the Milgroom et al. (2014) or Zhang et al. (2019) studies, or herein. Bootstrap values are indicated on branches but values below 0.75 were removed for image clarity

Results and discussion

In pathology assays isolate BRIP 71181 showed a severe disease phenotype on cotton plants (Fig. 1) and was identified as being a defoliating strain based on a PCR assay (Supplementary Fig. 1). In addition to cotton this isolate is able to infect *Nicotiana benthamiana* (Chen et al. 2024).

Although isolate BRIP 71172 was isolated from a diseased cotton plant, it has not been tested for virulence towards cotton.

The genomes of the two Australian *V. dahliae* isolates were consistent in size (~31–34 Mbp) (Table 1) with that reported for other released genomes of this species (Klosterman et al. 2011; de Jonge et al. 2012). BUSCO analysis suggested that the genomes encoded 93–95% of all conserved eukaryotic genes (Table 1). As a point of reference the genome of *V. dahliae* isolate VdLs.17 which has been sequenced telomere-to-telomere returned a BUSCO statistics of 94.6% complete, with duplicated, fragmented or missing percentages of 0.2, 1.5 and 3.9, respectively. Thus the two genomes sequenced here are very close to capturing most of the conserved gene space of *V. dahliae*. To determine how the Australian isolates related to other isolates of *V. dahliae* a genome wide analysis based on 17,238 polymorphic sites across a large population of *V. dahliae* and one *V. alfalfae* isolate were used to build a phylogenetic tree (Fig. 2, Supplementary Data 2). This analysis positioned BRIP 71181 with defoliating strains in lineage 1 A/1B. Isolate BRIP 71172 grouped with lineage 2 A isolates that are classified as non-defoliating towards cotton. Isolate BRIP 71172, but not BRIP 71181, encodes copies of both *Ave1* and *Av2* genes that are identical to that in other race 1 strains sequence. *Ave1* encodes a secreted protein that is recognised by the tomato Ve1 resistance gene product and hence its expression governs classification of strains as race 1 (de Jonge et al. 2012). *Av2* also encodes an avirulence protein recognised by the V2 resistance gene product from tomato (Chavarro-Carrero et al. 2021). Although *V. dahliae* is considered an asexual pathogen, isolates contain one of two mating types, although there is an extreme skew towards *MAT1-2* (Zhang et al. 2021 and references therein; Milgroom et al. 2014). Both BRIP 71172 and BRIP 71181 contained the *MAT1-2* locus.

Recently Chen et al. (2018) defined a genomic region that was involved in the high levels of virulence of defoliating strains of *V. dahliae*. This region was identified through population genomics, which compared isolates in the $2B^{824}$ lineage with those in the 1 A/1B lineage (Zhang et al. 2019). Zhang et al. (2019) went on to provide strong evidence for this region's role in defoliation, through knockouts and complementation experiments (Zhang et al. 2019) However, based on alignment of genomic reads to the Vd991 defoliating strain genome, this region appears to be absent from both the BRIP 71181 (VCG1A/B presumed defoliating) and BRIP 71182 (VCG2A presumed non-defoliating) strains from Australia assessed here (Fig. 3). Together with the highly aggressive nature of BRIP 71181 and its placement in VCG1A/B, this highlights that the defoliation phenotype expressed by many strains of *V. dahliae* may be genetically more complicated than a simple single locus encoded trait.

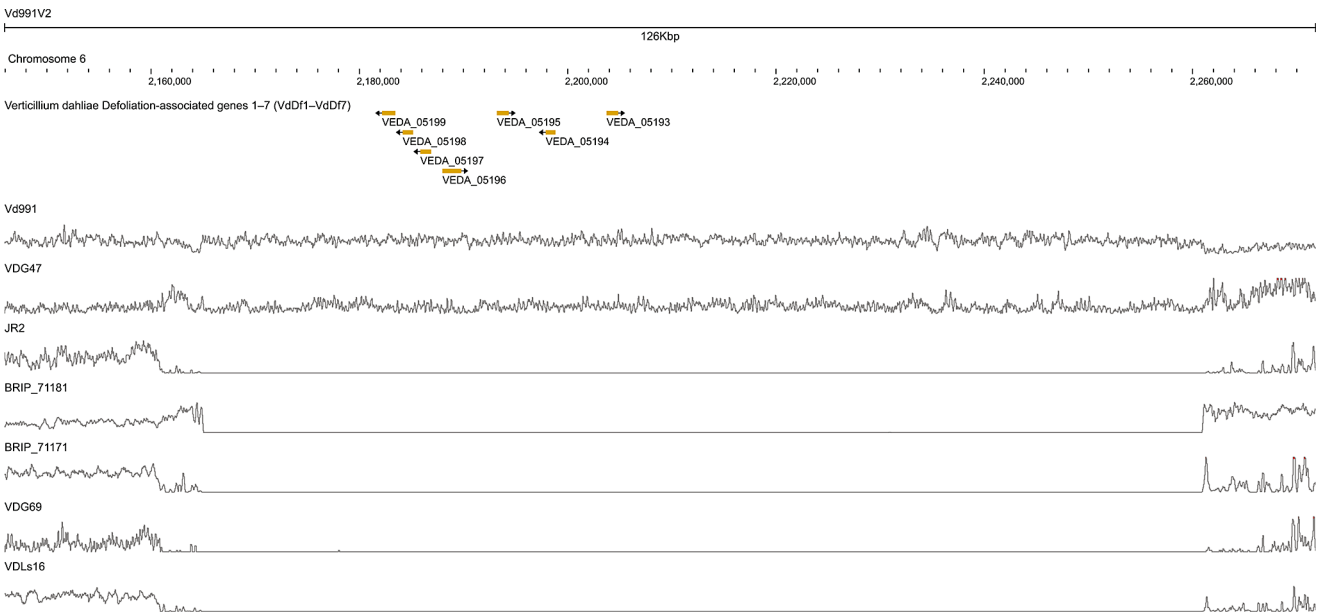

Fig. 3 Sequence coverage across the lineage specific region on chromosome 6 of Verticillium dahliae previously identified as being responsible for the defoliation phenotype on cotton. A 126 Kbp region of chromosome 6 from isolate Vd991 is represented and the seven genes that have been defined as defoliation-associated are displayed in yellow. Coverage graphs are represented by line graphs. Due to differences in read depth with the separate isolates the y-axis for all line graphs has been auto-scaled but the lower bound in all cases is zero. The flat lines observed for isolate JR2, BRIP 71181, BRIP 71171, VDG69 and VdLs16 are indicative of this region being absent from these isolates, in contrast to being present in Vd991 and VDG47. This analysis made use of the defoliating strains (Vd991 and VDG47) and non-defoliating strains (VDG69, JR2 and VdLs16) characterised by Zhang et al. (2019)

The genomes presented here provide a basis to begin to understand the genomic variability present in Australian strains of *V. dahliae* in the context of the global populations of this important pathogen. Although the two Australian strains phylogenetically group with either the 2 A–1 A/1B lineages, there are clear differences in the genome of isolate BRIP 71181 and the defoliating strains from China.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13313-024-00993-5.

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Data availability The raw sequence reads, assembly and annotation are available under the NCBI BioProject PRJNA453648 and PRJNA506278.

Declarations

Ethical approval Not applicable.

Conflict of interest The authors have no conflict of interest to declare that are relevant to this article.

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