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SHORT COMMUNICATION

Chromosome-Scale Genome Sequence of *Alternaria alternata* Causing Alternaria Brown Spot of Citrus

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Alternaria brown spot (ABS), caused by *Alternaria alternata*, is an economically important fungal disease of citrus worldwide. The ABS pathogen *A. alternata* tangerine pathotype can produce a host-specific ACT toxin, which is regulated by ACT toxin gene cluster located in the conditionally dispensable chromosome (CDC). Previously, we have assembled a draft genome of *A. alternata* tangerine pathotype strain Z7, which comprises 165 contigs. In this study, we report a chromosome-level genome assembly of *A. alternata* Z7 through the combination of Oxford Nanopore sequencing and Illumina sequencing technologies. The assembly of *A. alternata* Z7 had a total size of 34.28 Mb, with a GC content of 51.01% and contig N_{50} of 3.08 Mb. The genome is encompassed 12,067 protein-coding genes, 34 ribosomal RNAs, and 107 transfer RNAs. Interestingly, *A. alternata* Z7 is composed of 10 essential chromosomes and 2 CDCs, which is consistent with the experimental evidences of pulsed-field gel electrophoresis. To our best knowledge, this is the first chromosome-level genome assembly of *A. alternata*. In addition, a database for citrus-related *Alternaria* genomes has been established to provide public resources for the sequences, annotation and comparative genomics data of *Alternaria* spp. The improved genome sequence and annotation at the chromosome level is a significant step toward a better understanding of the pathogenicity of *A. alternata*. The database will be updated regularly whenever the genomes of newly isolated *Alternaria* spp. are available. The citrus-related *Alternaria* genomes database is open accessible through the Citrus Fungal Disease Database.

Keywords: *Alternaria alternata*, Alternaria brown spot, fungal pathogen, microbial genome sequencing

The *Alternaria alternata* species group (*Alternaria* section *Alternaria*; *Alternaria alternaria* (Fr.) Keissl.) consists of economically important filamentous fungi with a wide host range (Tsuge et al. 2013; Woudenberg et al. 2015). *A. alternata* is composed of at least 11 pathotypes, each of which can produce a unique host-selective toxin that kills host cells before invasion and extensively absorbs nutrients from dead tissues (Akimitsu et al. 2014; Thomma 2003). In citrus plants, there are two distinct pathotypes: (i) *A. alternata* tangerine pathotype, which produces a host-specific *Alternaria citri* toxin (ACT toxin), causing Alternaria brown spot (ABS) of tangerine (*Citrus reticulata* Blanco), grapefruit (*C. paradisi* Macfad.), hybrids of tangerine and grapefruit, and hybrids of tangerine and sweet orange (Chung 2012; Huang et al. 2015); and (ii) *A. alternata* rough lemon pathotype, which produces *A. citri* rough lemon (ACRL toxin), causing leaf spot of rough lemon and Rangpur lime (Tsuge et al. 2013). ABS mainly infects young leaves, young shoots, and young fruit of susceptible citrus, causing small, circular to oval, light-brown to dark-brown necrotic lesions with an obvious yellow halo around the lesion of diseased citrus leaves (Huang et al. 2015; Wang et al. 2010). Interestingly, the production of ACT toxin is regulated by several ACTT genes (*ACTT1*, *ACTT2*, *ACTT3, ACTT5*, *ACTT6, ACTTS2*, and *ACTTS3*) located in the conditionally dispensable chromosome (CDC) (Hatta et al. 2002; Wang et al. 2019). Genetic inactivation of ACTT gene will block the biosynthesis of ACT toxin, leading to complete loss of virulence (Ajiro et al. 2010; Miyamoto et al. 2010).

Citrus is the largest subtropical and tropical fruit crop in the world and is cultivated on over 9.28 million ha, with an annual production of 148 million metric tons. The necrotroph *A. alternata* is an important citrus fungal pathogen with global distribution. In 1903, ABS was first reported in Australia on Emperor mandarin (Cobb 1903). Because of its morphological similarity to the pathogen of Citrus black rot, the causal agent of Alternaria brown rot was

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initially identified as *Alternaria citri* Ellis & Pierce. However, *A. citri* was found to be misidentified in 1983 and was reclassified as *A. alternata* based on the morphological characteristics (Nishimura and Kohmoto 1983). To date, ABS has been reported in the United States (Whiteside 1976), Israel (Solel 1991), Turkey (Canihos et al. 1997), South Africa (Swart et al. 1998), Spain (Vicent et al. 2000), Brazil, Argentina (Peres et al. 2003), Greece (Elena 2006), Peru (Marín et al. 2006), Colombia, Iran (Kakvan et al. 2012), and Italy (Aiello et al. 2020), and poses a serious threat to the citrus industry. The pathogen *A. alternata* is able to overwinter in plant residues and soils in the citrus orchard. The spots gradually become round or irregular and are uniformly distributed on the citrus leaves, causing severe defoliation and leaf chlorosis. After defoliation, the hypha in the fallen leaves can produce a large number of conidia, which are widely spread by air currents or rain splashes, and continue to cause further infections (Huang et al. 2015; Li et al. 2015). China is one of the largest citrus-producing and -consuming countries in the world, with a cultivated area of 2.49 million ha and an annual production of 41.38 million metric tons in 2018. ABS caused by *A. alternata* on citrus was first observed in Wenshan, Yunnan Province, southern China in 2010 (Wang et al. 2010). Subsequently, ABS was observed in Chongqing, Zhejiang, Hunan, Yunnan, Guangdong, Guangxi and Sichuan Provinces, causing serious losses in citrus production (Huang et al. 2015). Without proper management, ABS infection can lead to serious leaf spots, early leaf defoliation, shoot dieback, and early fruit drop. The management of ABS in China, the largest citrus-producing country worldwide, relies heavily on the use of fungicide (Li et al. 2015). However, the extensive use of chemical agents not only causes serious pesticide residues in citrus but also potentially threatens human health, and also causes serious ecological and environmental safety problems (Li et al. 2015).

The use of long reads derived from high-throughput sequencing is a powerful approach to produce genome assembly of fungal genomes. To date, the genomes of various *Alternaria* spp., including *A. alternata*, have been sequenced in the past few years. The available draft genome of *A. alternata* includes *A. alternata* apple pathotype FERA1166 and *A. alternata* Japanese Pear pathotype FERA650 (Armitage et al. 2020; Dang et al. 2015). These genome sequences indeed provide new insight into the species classification, gene biological function, molecular identification, and evolution of *Alternaria* spp. However, most of these genome assemblies are highly fragmented because they were generated using short-read sequencing technologies alone or combined with the PacBio long reads. Therefore, it is difficult to distinguish genomic fragments from essential chromosomes (ECs) and CDCs. Previously, we have assembled a draft genome of *A. alternata* tangerine pathotype Z7, which contains 165 contigs (GenBank accession number GCA_001572055.1) (Wang et al. 2016, 2019). This assembly provides evidence on the identity of Z7 in terms of taxonomical classification (Huang et al. 2015) and has guided multiple biological studies in our lab to address important questions regarding the virulence and regulation of ACT production of Z7 (Fu et al. 2020; Gai et al. 2021; Ma et al. 2019; Wang et al. 2018).

The fungus was grown on potato dextrose broth (PDB) medium (200 g of potato, 20 g of glucose, and 1 liter of purified water) on a rotary shaker set at 180 rpm at 25°C. For DNA extraction, the mycelia produced in PDB was filtered and ground into fine powder with liquid nitrogen and stored at −80°C. High molecular weight genomic DNA was extracted from 3-day-old Z7 mycelia using the cetyltrimethylammonium bromide method. Illumina HiSeq and Oxford Nanopore technologies were used to generate 7.87 Gb of paired-end short sequence reads and 13.28 Gb of long reads, respectively. The 1627,160 Oxford Nanopore reads (386× coverage) have a read length N_{50} of 28,902 bp after filtering. We assembled the Z7 genome from Oxford Nanopore reads using the assembler Nanopore Error Correction and Assembly Tool (NECAT) with default parameters and polished the assembly with Illumina PE reads using Pilon v1.23 (Walker et al. 2014). The final Z7 assembly included 13 contigs with a total genome size of 34,280,341 bp (Table 1). Benchmarking universal single-copy ortholog (BUSCO) v5.0 β with the 'fungi_odb10' library was used to evaluate the completeness of genome assembly (Simão et al. 2015). Results showed that the genome completeness (BUSCO complete + partial) of *A. alternata* strain Z7 was 97.9%. Other statistics of the genome of *A. alternata* strain Z7 are shown in Tables 1 and 2.

The transcriptome profiling was performed for *A. alternata* Z7 and the *AaMetR* disrupted mutant (Gai et al. 2019). After trimming low-quality sequences, the cleaned RNA-sequencing (RNA-seq) reads were mapped to the reference genome of *A. alternata* Z7 with hisat2 (version 2.1.0) (Kim et al. 2019), using the following settings: '−p 42 –min-intronlen 20 –max-intronlen 4000'. The resulting alignments were converted into the binary format with SAMtools (Li et al. 2009), and the binary alignment files were converted to hint files with bam2hints (with the parameter '–introns only') from AUGUSTUS (version 3.3.0) (Hoff and Stanke 2019). De novo assembly of the transcriptome was performed with the cleaned RNA-seq reads by rnaSPAdes v3.13 (Bushmanova et al. 2019) and Trinity v2.11.0 (Grabherr et al. 2011) with default parameters. For gene prediction and annotation, the genomes were first annotated using a Perl script gmes_petap.pl from the GeneMark-ET v4.33 (Brůna et al. 2020), with the RNA-seq files as biological evidence. The resulting general feature format (gff) files were used for the protein model training of AUGUSTUS v3.3 (Hoff and Stanke 2019). In addition to GeneMark-ET and AUGUSTUS, the genomes were also annotated using SNAP v2013.02.16. For redundant transcripts and alternative splicing prediction, the transcript reads were mapped to the reference genome by a Perl script Launch_PASA_pipeline.pl from the Program to Assemble Spliced Alignments (PASA) v2.2.0 annotation pipeline (Campbell et al. 2006). For gene prediction by homolog protein, the genomes were annotated by a Perl script homolog_genewise from the GeneWise v2.4.1 (Birney et al. 2004). The software RepeatMasker v4.0.5 was used to screen DNA sequences for interspersed repeats and low complexity DNA sequences. RepeatModeler v1.0.8 was used to identify the transposable elements families. The genome annotation pipeline MAKER (version 2.31.9) (Cantarel et al. 2008) was used to perform genome annotation, with the transcripts as expressed sequence tag

Table 1. Summary of the genome assembly and annotation statistics of *Alternaria alternata* Z7 (GenBank accession GCA_014751505.1) compared with a previous assembly of Z7 (GenBank accession GCA_001572055.1) and *A. solani* CBS_143772 (GenBank accession GCA_001572055.1)[a]

Strains	Z7 (this study)	Z7 (GCA_001572055.1)	*A. solani* altNL03003
Number of contigs	12	165	10
Number of chromosomes	12	NA	10
Number of CDC	2	NA	0
Genome size (Mb)	34.35	34.35	32.77
GC content (%)	50.99	50.98	51.32
Contig N50 (bp)	3,085,453	1,128,165	2,866,555
Contig N90 (bp)	2,401,432	184,938	2,309,181
Number of protein-coding genes	12,067	12,048	NA
Genome completeness (%)[b]	97.9	98.1	97.9

[a] *A. alternata* Z7 = *A. alternata* CGMCC3.18907 and *A. solani* CBS_143772 = *A. solani* altNL03003.

[b] Benchmarking universal single-copy ortholog (BUSCO) complete + partial.

evidence, the homolog proteins as homology evidence, and the transposable elements as repeat masking. Consequently, gene predictions generated by different software were fed to EVidenceModeler version 1.1.1 to select the best models (Haas et al. 2008), which identified 12,067 protein-coding genes, 34 ribosomal RNAs, and 107 transfer RNAs (Tables 1 and 2).

Carbohydrate-active enzymes (CAZYmes) play a vital role in destroying host cell wall components to establish a successful infection process. The dbCAN v6.0 database (Lombard et al. 2014) was used to predict CAZYmes by using hmmscan, and showed 820 CAZYmes (6.80% of the proteome), which included 294 glycosyl hydrolases, 155 carbohydrate esterases, 110 glycoside transferases, 75 carbohydrate-binding modules, 161 auxiliary activities, and 25 polysaccharide lyases. The UniProtKB/Swiss-Prot database is a high-quality annotated and nonredundant protein sequence database, which brings together experimental results, computed features, and scientific conclusions. The proteins were functionally annotated by using Protein-Protein BLAST v2.6.0 against the Swiss-Prot database (Bairoch and Apweiler 2000) with the parameters '–evalue 1e-5 –max_target_seqs 20', which resulted in a best-hit description for 7,907 genes representing 65.52% of the proteome. The pathogenicity genes were predicted by using BLASTP against the Pathogen-Host Interaction (PHI) database with a strict threshold of Match ≥ 50% and Identity ≥ 50% (Winnenburg et al. 2006), which identified 883 putative PHI genes (7.31% of the proteome). SignalP v5.0 analysis of the proteomes of *A. alternata* Z7 revealed that 1,331 predicted proteins (representing 11.03% of the proteome) contain a secretion signal peptide (Armenteros et al. 2019). Secondary metabolites (SMs) play an essential role in the survival of fungi in their ecological niche. Fungal SM-related gene clusters were predicted by the online tool antiSMASH 5.0 (Blin et al. 2019), which identified 31 SM clusters (6 nonribosomal peptides synthetase [NRPS], 9 type 1 [T1] polyketide synthase [PKS], 7 terpene, 7 NRPS-like, 1 fungal-RiPP, and 1 T3 PKS). The R genoplotR package was used to visualize the SM gene clusters in the genome of *A. alternata* Z7 (Fig. 1).

For comparative genomics analysis, the genomes of *A. solani* CBS_143772 (tomato early blight, GenBank accession GCA_002952155.1), *A. gaisen* FERA_650 (black spot of Japanese pear, GenBank accession GCA_004156025.2), *A. alternata* JS-0527 (an endophytic fungus isolated from a leaf tissue of *Phragmites australis*, GenBank accession GCA_011420255.1), *A. tenuissima* FERA 1166 (isolated from infected leaves of apple, GenBank accession GCA_004156035.1), and *A. alternata* NAP07 (isolated from diseased lesions of peach, GenBank accession GCA_009932595.1) were collected and compared with the *A. alternata* Z7 (tangerine pathotype) genome using all-vs-all BLAST search with a strict threshold '–evalue 1e-5 –outfmt 6'. Alignments with cumulative sizes smaller than 2 kb were removed and the filtered alignments were clustered into syntenic blocks. The macrosynteny plots were constructed using the R genoplotR package (Guy et al. 2010). The result showed remarkably syntenic and collinear relationships among different *Alternaria* genomes (Fig. 2). Interestingly, we found that *A. alternata* Z7 is composed of 10 ECs and 2 CDCs (GenBank accession numbers CP061875.1 to CP061886.1), which is consistent with previous experimental evidence generated by pulsed-field gel electrophoresis (Wang et al. 2019). We also found that most *Alternaria* spp. have 10 essential chromosomes, although some of these may have undergone rearrangements. Interestingly, we did not find homologs of CDCs in the endophytic fungus *A. alternata* JS-0527, concordant with the fact that CDCs play a vital role in the pathogenicity of *A. alternata*.

To investigate the species similarity of *Alternaria*, the nucleotide-level genomic similarity of 27 *Alternaria* spp. were analyzed by pyANI and visualized by the R pheatmap package. In the average nucleotide identity (ANI) heatmap, *A. alternata* strains ATCC11680, ATCC66891, BMP0270, JS-0527, FERA1177, B2a, JS-1623, SRC1lrK2f, and Z7 showed more than 98% ANI (Fig. 3). We found that *A. gaisen*, also known as *A. alternata* Japanese pear pathotype, has more than 98% ANI similarity with *A. fragariae* and 96% ANI similarity with *A. alternata*. Furthermore, we found that the ANI of *A. tenuissima, A. turkisafria, A. limoniasperae, A. mali, A. citriarbusti, Alternaria* sp. MG1, and *A. alternata* showed more than 98% similarity, which is congruent with multigene phylogenies based on commonly used gene regions (Woudenberg et al. 2015).

To our knowledge, this is the first report of the chromosome-level genome assembly of *A. alternata* using the Illumina and Oxford Nanopore sequencing platforms. The assembly had a total genome size of 34.28 Mb, and the contig N_{50} reached 3.08 Mb. In total, 12,067 protein-coding genes were predicted, and 7,907 genes (65.52%) have been annotated. Macrosynteny analysis of the chromosome-level genome of *Alternaria* spp. showed syntenic and collinear relationships among different *Alternaria* genomes. The genome sequence of *A. alternata* Z7 reported here is a significant improvement over previous genome assembly. We also performed comparative genomics analysis and found highly differentiated genomes among *A. alternata* strains, which will be valuable in further study of the preferential virulence to its original host plants. The genomic data in this study offer a valuable resource for facilitating the comparative genomics of *Alternaria* spp. as well as for studying the molecular basis of the pathogenicity of *A. alternata*.

Table 2. Genomic features of *Alternaria alternata* Z7 (GenBank accession GCA_014751505.1)[a]

ID[b]	Genome size (bp)	GC content (%)	Protein-coding genes	tRNA genes[c]	Gene density (genes/Mb)	Average length of gene (bp)	Average number of exons/gene	GenBank accession
Chr 1	6,770,053	51.19	2,446	38	361	1,955	2.55	CP061875.1
Chr 2	5,542,836	51.01	2,052	26	370	1,882	2.47	CP061876.1
Chr 3	3,269,484	51.06	1,193	22	365	1,864	2.49	CP061877.1
Chr 4	3,085,453	51.04	1,124	9	364	1,956	2.55	CP061878.1
Chr 5	2,863,349	50.88	1,003	10	350	1,960	2.59	CP061879.1
Chr 6	2,544,158	50.96	877	22	345	1,938	2.45	CP061880.1
Chr 7	2,519,108	50.89	868	16	345	1,995	2.59	CP061881.1
Chr 8	2,464,928	51.06	861	9	349	1,843	2.45	CP061882.1
Chr 9	2,401,432	50.91	820	17	341	2,023	2.55	CP061883.1
Chr 10	1,841,737	51	628	13	341	1,976	2.5	CP061884.1
CDC 1	561,391	50.3	121	0	216	1,651	2.07	CP061885.1
CDC 2	416,412	50.4	74	0	178	1,663	2.27	CP061886.1

[a] A 75,279-bp contig was the mitochondrion of *Alternaria alternata* Z7.
[b] Chr = chromosome and CDC = conditionally dispensable chromosome.
[c] tRNA = transfer RNA.

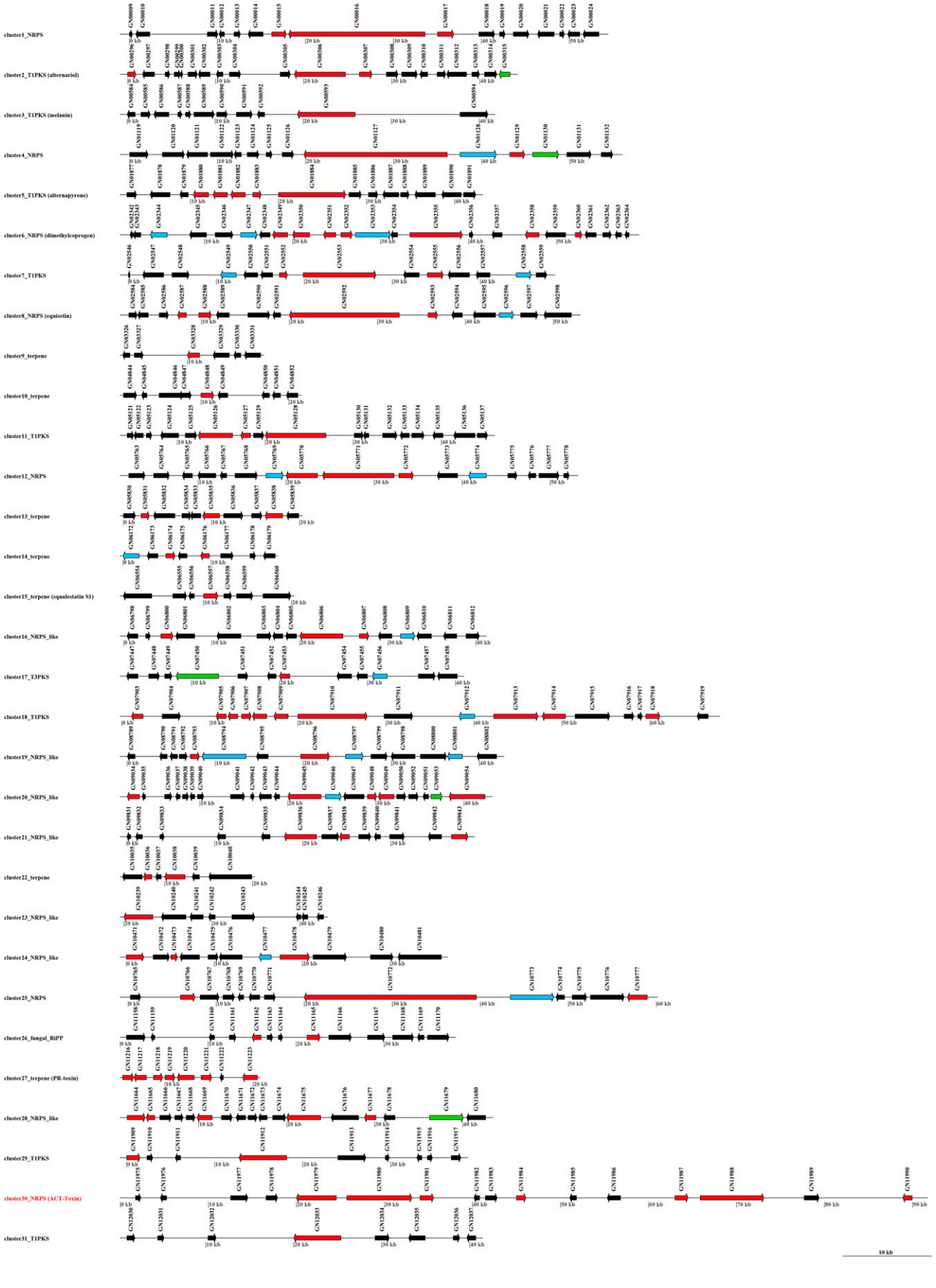

Fig. 1. Secondary metabolite gene clusters of *Alternaria alternata* Z7. Abbreviations: PKS = polyketide synthase, NRPS = nonribosomal peptides synthetase, T1 = type 1, and terpene = terpene synthetase.

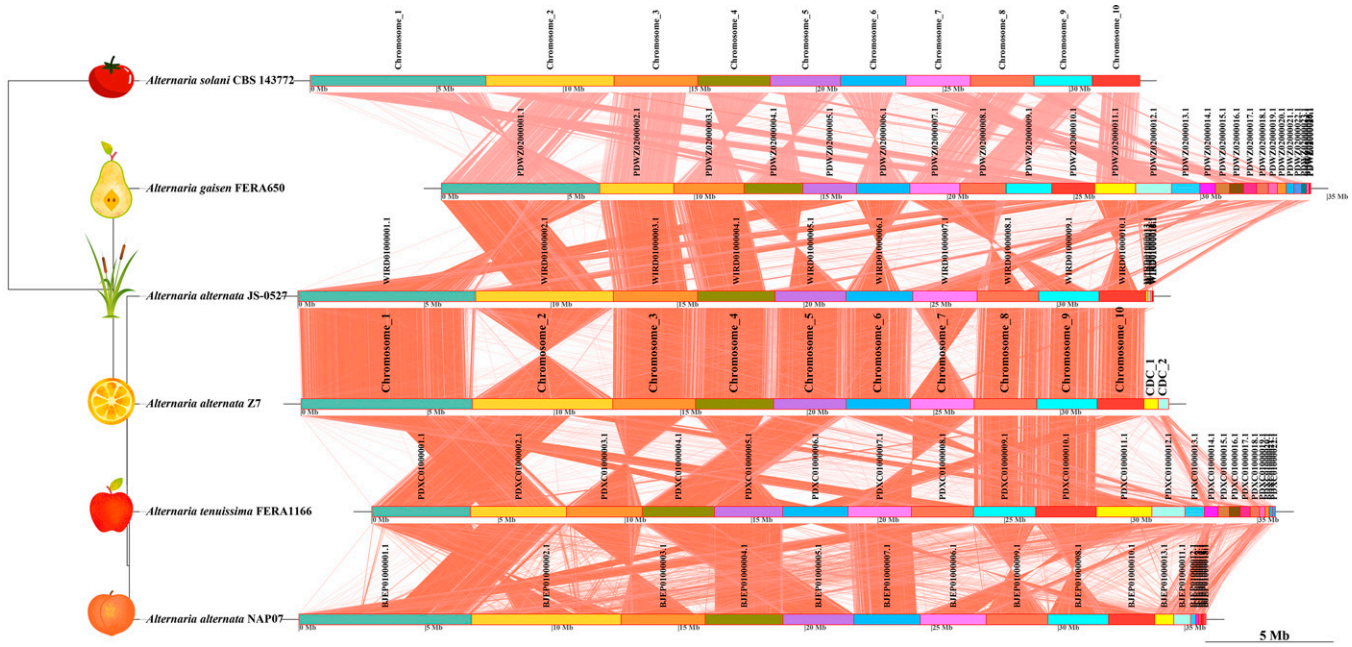

Fig. 2. Synteny and collinearity analysis of genome of *Alternaria alternata* Z7 (GenBank accession GCA_014751505.1) and five *Alternaria* genomes using NCBI BLASTN. The phylogenetic tree with genome alignment was generated by the R GenoPlotR package.

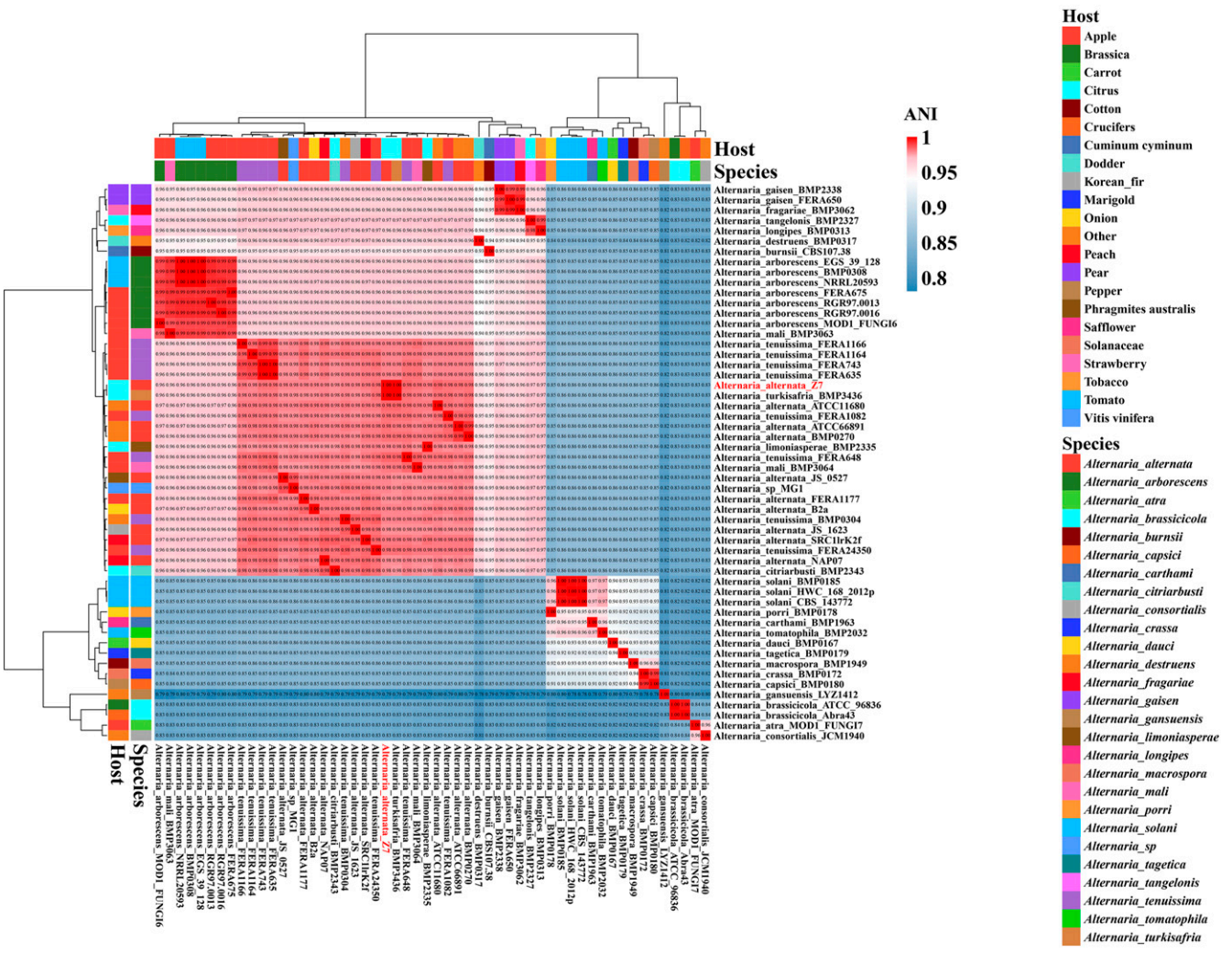

Fig. 3. Heatmap showing the average nucleotide identity (ANI) relationships among 54 *Alternaria* genomes that span the diversity of different *Alternaria* spp., including *Alternaria alternata*. The ANI heatmap was generated by the R Pheatmap package.

Data availability.

This Whole-Genome project has been deposited at NCBI BioProject under the accession PRJNA661160. The chromosomal sequence and gene annotation of Z7 can be found at GenBank with accession numbers to GCA_014751505.1. Oxford Nanopore reads, Illumina reads, and RNA-seq Illumina data are available in the NCBI BioSample under the accession numbers SAMN16047574, SAMN16047331, and SAMN15746765, respectively. The whole-genome sequences are available at GenBank under the accession number CP061875.1 for chromosome 1, CP061876.1 for chromosome 2, CP061877.1 for chromosome 3, CP061878.1 for chromosome 4, CP061879.1 for chromosome 5, CP061880.1 for chromosome 6, CP061881.1 for chromosome 7, CP061882.1 for chromosome 8, CP061883.1 for chromosome 9, CP061884.1 for chromosome 10, CP061885.1 for CDC 1, and CP061886.1 for CDC 2. The SM biosynthesis clusters of *A. alternata* Z7 are available online. The whole-genome and annotation data are also available at the ZJUDATA website.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

A. alternata Z7: http://www.zjudata.com/alternaria/Z7/
Alternaria genomes database: http://www.zjudata.com/alternaria/blast.php
ANI Calculator: http://enve-omics.ce.gatech.edu/ani/
antiSMSH fungal version: https://fungismash.secondarymetabolites.org/
Benchmarking Universal Single-Copy Orthologs (BUSCO): https://busco.ezlab.org/
Carbohydrate-Active enZYmes Database: http://www.cazy.org/
Citrus Fungal Disease Database: http://www.zjudata.com/
NECAT: https://github.com/xiaochuanle/NECAT
NCBI BioProject accession PRJNA661160: https://www.ncbi.nlm.nih.gov/bioproject/661160
Pathogen-Host Interaction (PHI) database: http://www.phi-base.org/
pyANI: http://huttonics.github.io/pyani/
RepeatMasker v4.0.5: http://repeatmasker.org/
RepeatModeler v1.0.8: http://www.repeatmasker.org/RepeatModeler/
SignalP-5.0 Server: http://www.cbs.dtu.dk/services/SignalP/
SNAP v2013.02.16: http://ftp.debian.org/debian/pool/main/s/snap/
ZJUDATA website: http://www.zjudata.com/alternaria/blast.php

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