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Short Communication

The Genome Sequence of the Citrus Melanose Pathogen *Diaporthe citri* and Two Citrus-Related *Diaporthe* Species

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ABSTRACT

Melanose disease is one the most widely distributed and economically important fungal diseases of citrus worldwide. The causative agent is the filamentous fungus *Diaporthe citri* (syn. *Phomopsis citri*). Here, we report the genome assemblies of three strains of *D. citri*, namely strains ZJUD2, ZJUD14, and Q7, which were generated using a combination of PacBio Sequel long-read and Illumina paired-end sequencing data. The assembled genomes of *D. citri* ranged from 52.06 to 63.61 Mb in genome size, containing 15,977 to 16,622 protein-coding genes. We also sequenced and annotated the genome sequences of two citrus-related *Diaporthe* species, *D. citriasiana* and *D. citrichinensis*. In addition, a database for citrus-related *Diaporthe* genomes was established to provide a public platform to access genome sequences, genome annotation and comparative genomics data of these *Diaporthe* species. The described genome sequences and the citrus-related *Diaporthe* genomes database provide a useful resource for the study of fungal biology, pathogen–host interaction, molecular diagnostic marker development, and population genomic analyses of *Diaporthe* species. The database will be updated regularly when the genomes of newly isolated *Diaporthe* species are sequenced. The citrus-related *Diaporthe* genomes database is freely available for nonprofit use at zjudata.com/blast/diaporthe.php.

Keywords: citrus melanose, citrus-related *Diaporthe* species, *Diaporthe citri*, fungal pathogen, microbe-genome sequencing

The genus *Diaporthe* includes saprophytic, endophytic, and pathogenic-plant parasites with a wide range of hosts, including many economically important crops (Chaisiri et al. 2020; Gomes et al. 2013; Guarnaccia and Crous 2017). Citrus melanose, caused by *Diaporthe citri* Wolf (syn. *Phomopsis citri* H.S. Fawc.), is one of the most destructive and widespread citrus fungal diseases in the world. Melanose disease can infect almost all citrus varieties, including mandarin (*Citrus reticulata* Blanco and *C. unshiu* Marc.), orange (*C. sinensis* Osbeck), pomelo (*C. maxima* (Burm) Merr.), kumquat (*Fortunella margarita* (Lour.) Swingle), grapefruit (*C. paradisi* Macf.), and lemon (*C. limon* (L.) Burm. f.) (Chaisiri et al. 2020; Huang et al. 2013). *D. citri* can cause a variety of symptoms, such as stem-end rot and melanose of citrus fruits, young leaves and shoots, as well as blight of perennial branches and trunks (Huang et al. 2013; (Jiang et al. 2012; Udayanga et al. 2014). In 1912, *D. citri* was first described as the causal agent of citrus stem-end rot in Florida, U.S.A., and was subsequently recorded in major citrus-producing countries around the world (Fawcett 1912; Huang et al. 2013). In addition to *D. citri*, more than 20 *Diaporthe* species, including *D. citriasiana*, *D. citrichinensis*, *D. cytosporella*, *D. foeniculina*, *D. discoidispora*, *D. eres*, *D. sojae*, and *D. unshiuensis* are also occasionally isolated from citrus, although the ecological habits of these species are still unknown (Chaisiri et al. 2020; Gomes et al. 2013; Huang et al. 2013; Udayanga et al. 2014).

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Although *Diaporthe* is an economically important genus worldwide, its genomic resources are still poor. Until now, there are only seven *Diaporthe* species, including *D. longicolla* (Li et al. 2015, 2017), *D. aspalathi* (Li et al. 2016), *D. ampelina* (Bhargavi et al. 2018; Savitha et al. 2016), *D. helianthi* (Baroncelli et al. 2016), *D. capsici* (Fang et al. 2020), *D. phragmitis* NJD1 (Wang et al. 2021), and *Diaporthe* sp. strain HANT25 (Tulsook et al. 2020) with available genome sequences. As mentioned above, citrus melanose caused by *D. citri* is one of the most important fungal diseases of citrus, posing a serious threat to citrus production. Therefore, genome sequencing can provide useful resources for further investigation on the molecular mechanism of fungicide resistance, pathogen–host interaction, and population genome-related research of this relevant plant pathogen.

The *D. citri* strain ZJUD2 (deposited at Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands with the accession number CBS134237) was isolated from stem end rot infected citrus (*C. reticulata*) collected from Lishui, Zhejiang Province, China in 2009. *D. citri* strain ZJUD14 was isolated from melanose infected citrus fruits collected from Xiangxi, Hunan Province, China in 2011. *D. citri* Q7 was isolated from infected citrus fruits collected from Quzhou, Zhejiang Province, China in 2015. Among the three *D. citri* strains, ZJUD2 and Q7 belong to mating-type II (MAT1-2), and ZJUD14 belongs to *D. citri* mating-type I (MAT1-1). In addition to *D. citri*, two citrus-related *Diaporthe* species, *D. citriasiana* ZJUD30 and *D. citrichinensis* ZJUD34, were isolated from citrus stems (*C. unshiu*) collected from Chenggu, Shaanxi Province, China. The taxonomic position of these *Diaporthe* strains have been identified through phylogenetic analysis of rDNA internal transcribed spacer, elongation factor, β-tubulin, and calmodulin genes (Huang et al. 2013).

For DNA extraction, fungi were grown on PDB (potato dextrose broth medium without agar) at 25°C on a rotary shaker with a rotation speed of 180 rpm. The mycelia produced was filtered and ground into fine powder with liquid nitrogen. Genomic DNA was

extracted from the 5-day cultured mycelia using the Biospin Fungus Genomic DNA Extraction Kit (Bio-Flux, Bioer Technology Co., China) according to the manufacturer's instructions. The preparation of a fragmented genomic DNA library was performed using a Nextera DNA sample preparation kit, according to the manufacturer's protocols (Illumina, San Diego, CA). Quality and quantity of each library was checked using a 2100 Bioanalyzer (Agilent Technologies). Sequencing was conducted in paired-end 2 × 150 bp mode on an Illumina HiSeq platform, according to the manufacturer's protocols (Illumina, San Diego, CA). The single-molecule real-time sequencing (SMRT) bell (SMRT Bell) library of *D. citri* Q7 was constructed using a PacBio DNA Template Prep Kit 1.0 (Pacific Biosciences) and was sequenced using the Sequel sequencing platform (Pacific Biosciences) based on the manufacturer's instructions. In total, 118,428 million raw pair-end reads (150 × 2) were generated on Illumina Hiseq platform. The PacBio Sequel platform produced a total of 3,546 Mb of high-quality genomic data. The average coverage of SMRT sequences on the genome of *D. citri* strain Q7 was 55-fold. The N50 lengths of subreads reached 13.9 kb, suggesting efficient production of ultra-long genomic sequences for the assembly. The raw Illumina reads were deposited in the sequence read archive (SRA) database of National Center for Biotechnology Information (NCBI) with accession number PRJNA660209 (https://www.ncbi.nlm.nih.gov/bioproject/660209). The assembly statistics of each *Diaporthe* Illumina data set are shown in Table 1.

The raw sequencing reads generated by Illumina Hiseq platform were processed to remove the adaptors and low-quality bases using Trimmomatic (version 0.36) with the following settings: 'PE -threads 24 ILLUMINACLIP:adapter.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:10 MINLEN:36 TOPHRED33' (Bolger et al. 2014). FastQC (version 0.11.6) was performed to check the quality of the cleaned Illumina data sets. Both the long-read PacBio Sequel and short-read Illumina sequencing datasets were used for genome assembly of *D. citri* Q7 with SPAdes v3.14, as this is a hybrid assembler (Bankevich et al. 2012). The genome sequences of ZJUD2, ZJUD14, ZJUD30, and ZJUD34 were assembled using Illumina Hiseq raw reads by SPAdes v3.14 with the parameter "-k 21,33,55,77,99,127–careful". Pilon v1.23 (Walker et al. 2014) was used to improve draft genome assemblies by filling gaps, fixing misassemblies and correcting bases. The genome sequences of *D. citri* ranged from 52.06 to 63.61 Mb, with N50 value of 235 to 1,223 kb and N90 value of 90 to 339 kb. The overall G + C content of *D. citri* ranged from 47.48 to 52.72%. The genome of *D. citriasiana* ZJUD30 is 52.39 Mb with an N50 of 189 kb. The genome of *D. citrichinensis* ZJUD34 is 54.5 Mb with an N50 of 573 kb. The completeness of assemblies was evaluated by BUSCO (benchmarking universal single-copy ortholog) v5.0 beta with 'fungi_odb9' library (Seppey et al. 2019). A total of 758 BUSCO groups were searched, and the genome sequences were estimated to be 98.30 to 99.20% complete, suggesting that these genomes are reliable for the downstream analyses. The statistics of each *Diaporthe* genome assembly are shown in Table 1.

Total RNA was extracted from the 5-day-old culture of *D. citri* Q7 using the AxyPrep Multi-Source Total RNA Miniprep Kit (Axygen, U.S.A.) according to the manufacturer's instructions. The quality and quantity of total RNA was evaluated using an Agilent Bioanalyzer (Agilent Technologies, CA, U.S.A.) and the Qubit 3.0 Fluorometer (Life Technologies, CA, U.S.A.). The transcriptome library was sequenced (150-bp paired-end reads) on the

TABLE 1. Genome annotation of citrus-related *Diaporthe* species in this study[a]

	D. citri	*D. citri*	*D. citri*	*D. citriasiana*	*D. citrichinensis*
Species	ZJUD2	ZJUD14	Q7	ZJUD30	ZJUD34
Mating type	MAT1-2	MAT1-1	MAT1-2	–	–
Location	Lishui, Zhejiang	Xiangxi, Hunan	Quzhou, Zhejiang	Chenggu, Shaanxi	Chenggu, Shaanxi
Host	*C. reticulata*	*C. reticulata*	*C. reticulata*	*C. unshiu*	*C. unshiu*
Raw reads number	26,737,151	28,454,959	25,114,535	18,770,319	19,351,211
Total bases	8,021,145,300 bp	8,536,487,700 bp	7,534,360,500 bp	5,631,095,700 bp	5,805,363,300 bp
Coverage (×)	130×	158×	173×	105×	106×
Contigs	91	360	168	750	178
Genome size	59,585,059 bp	52,062,681 bp	63,611,923 bp	52,393,681 bp	54,500,586 bp
The longest contig	2,920,297 bp	699,516 bp	2,350,144 bp	774,375 bp	2,305,902 bp
GC content	47.93%	52.76%	47.48%	52.04%	54.13%
Coding sequence	15,218	14,991	15,422	13,839	15,928
N50	1,223,719 bp	235,461 bp	1,063,595 bp	188,970 bp	573,128 bp
N90	339,329 bp	89,876 bp	226,254 bp	54,020 bp	181,392 bp
Complete BUSCOs (%)	98.50%	98.60%	98.50%	99.20%	98.30%
Duplicated BUSCOs (%)	0.5%	0.4%	1.8%	0.3%	0.9%
Fragmented BUSCOs (%)	0.9%	0.9%	0.9%	0.4%	0.9%
Missing BUSCOs (%)	0.6%	0.5%	0.6%	0.4%	0.8%
GenBank assembly accession	GCA_014872965.1	GCA_014872985.1	GCA_014873005.1	GCA_014872975.1	GCA_014872995.1
GenBank accession	JADAZQ000000000	JADAZP000000000	JADAZO000000000	JADAZS000000000	JADAZR000000000

[a] ZJUD represents *Diaporthe* strains collected and stored in Zhejiang University, China; CBS represents Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CGMCC represents China General Microbiological Culture Collection Center, China. ZJUD2, ZJUD30, and ZJUD34 are ex-type cultures: ZJUD2 = CBS134237; ZJUD30 = CBS134240 = CGMCC3.15224; and ZJUD34 = CBS134242 = CGMCC3.15225.

TABLE 2. Functional annotation of proteome of citrus-related *Diaporthe* species in this study[a]

Species	Strain	Coding sequence	SwissProt	PHI	SM clusters	SignalP5	GH	GT	PL	AA	CE	CBM	Biosample accession
D. citri	ZJUD2	15,218	9,449	1,231	98	1,860	505	217	65	418	281	94	SAMN15941434
D. citri	ZJUD14	14,991	9,517	1,269	98	1,837	514	210	62	407	286	102	SAMN15941435
D. citri	Q7	15,422	9,792	1,287	102	1,885	526	213	67	428	291	98	SAMN15944934
D. citriasiana	ZJUD30	13,839	8,998	1,223	88	1,643	483	217	57	379	269	104	SAMN15944717
D. citrichinensis	ZJUD34	15,928	10,190	1,282	110	2,034	547	213	58	437	326	121	SAMN15944718

[a] Pathogen–host interaction (PHI) database, secondary metabolite biosynthesis gene clusters (SM clusters), glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), auxiliary activities (AA), carbohydrate esterases (CE), and carbohydrate-binding modules (CBM).

Illumina HiSeq platform (Illumina, San Diego, CA, U.S.A.), which included nebulization and end repair of cDNA, ligation of adaptors, gel purification, PCR amplification and library purification. In total, ~31.261 million Illumina pair-end reads of 150-bp were generated from RNA-seq sequencing. After trimming low-quality sequences, de novo assembly was performed with cleaned reads by the RNA-Seq assembler Trinity (version 2.5.1) (Haas et al. 2013). Finally, RNA-seq reads were assembled into 55,308 transcripts, with an N50 of 2,120 bp. The average length of these transcripts was 2,435 bp, and the GC contents 56.48%. These transcripts ranged from 201 to 14,809 bp, with 20.12% longer than 1,000 bp.

For gene prediction and annotation, the cleaned RNA-seq reads were mapped to the reference genome with hisat2 (version 2.1.0) (Kim et al. 2015), using the following settings: "-p 42–min-intronlen 20–max-intronlen 4000". The resulting alignments were converted into binary format with SAMtools (Li et al. 2009), and the binary alignment files were processed with bam2hints (with parameter '–introns only') from AUGUSTUS (version 3.3.0) (Hoff and Stanke 2019). Gene predictions were performed using the gene prediction tool AUGUSTUS, with a training set of gene models generated by the self-training algorithm GeneMark-ES (version 4.3.3) (Brůna et al. 2020). The parameters of AUGUSTUS were "-strand=both, -genemodel=partial, -singlestrand=false, -protein=on, -introns=on, -start=on, -stop=on, -cds=on, -alternatives-from-evidence=true, and -gff3=on" and with assembled transcripts as biological evidence. A customized Perl script was used to further check the gene annotations of each *Diaporthe* genome predicated by AUGUSTUS to ensure no stop codons in the gene predictions. *D. citri* ranged from 14,991 to 15,422 protein-coding genes, with an average gene length of 1,663 to 1,691 bp. *D. citriasiana* ZJUD30 contains 14,272 protein-coding genes, with an average gene length of 1,683 bp. *D. citrichinensis* ZJUD34 contains 16,329 protein-coding genes, with an average gene length of 1,700 bp.

The proteomes of each *Diaporthe* strain were functionally annotated by using BLASTP against the SwissProt database, which found 9,449 to 10,190 genes annotated (62.09 to 65.02% of the proteome) (Bairoch and Apweiler 2000). The pathogenicity genes of *D. citri* were predicted by using BLASTP against the pathogen–host interaction (PHI) database (Winnenburg et al. 2006), which identified 1,231 to 1,287 putative PHI genes (8.08 to 8.46% of the proteome). The proteomes of *D. citri* were annotated using SignalP v5.0 and revealed that 1,837 ~1,885 predicted proteins (12.22 to 12.25% of the proteome) contain a secretion signal peptide (Almagro Armenteros et al. 2019). Carbohydrate-active enzymes play a vital role in destroying host cell wall components to establish a successful infection process. The dbCAN v6.0 database (Lombard et al. 2013) was used to predict carbohydrate-active enzymes (CAZymes) in *D. citri* by using hmmscan v3.0, which identified 505 to 526 glycoside hydrolases, 210 to 217 glycosyl transferases, 407 to 428 auxiliary activities, 281 to 291 carbohydrate esterases, 62 to 67 polysaccharide lyases, and 94 to 102 carbohydrate-binding modules. The CAZyme analysis identified 483 glycoside hydrolases, 217 glycosyl transferases, 57 polysaccharide lyases, 379 auxiliary activities, 269 carbohydrate esterases, and 104 carbohydrate-binding modules in the genome of *D. citriasiana* ZJUD30. The genome of *D. citrichinensis* ZJUD34 contain 547 glycoside hydrolases, 213 glycosyl transferases, 58 polysaccharide lyases, 437 auxiliary activities, 326 carbohydrate esterases, and 121 carbohydrate-binding modules. These results indicated that the numbers and types of CAZymes among different *Diaporthe* species are very similar. Secondary metabolites play an essential role in the survival of fungi in their ecological niche. We used the computational tool antiSMASH fungal version 5.0 (Blin et al. 2019) to perform the rapid genome-wide identification, annotation and analysis of secondary metabolite biosynthesis gene clusters (SM clusters) in different *Diaporthe* species. The

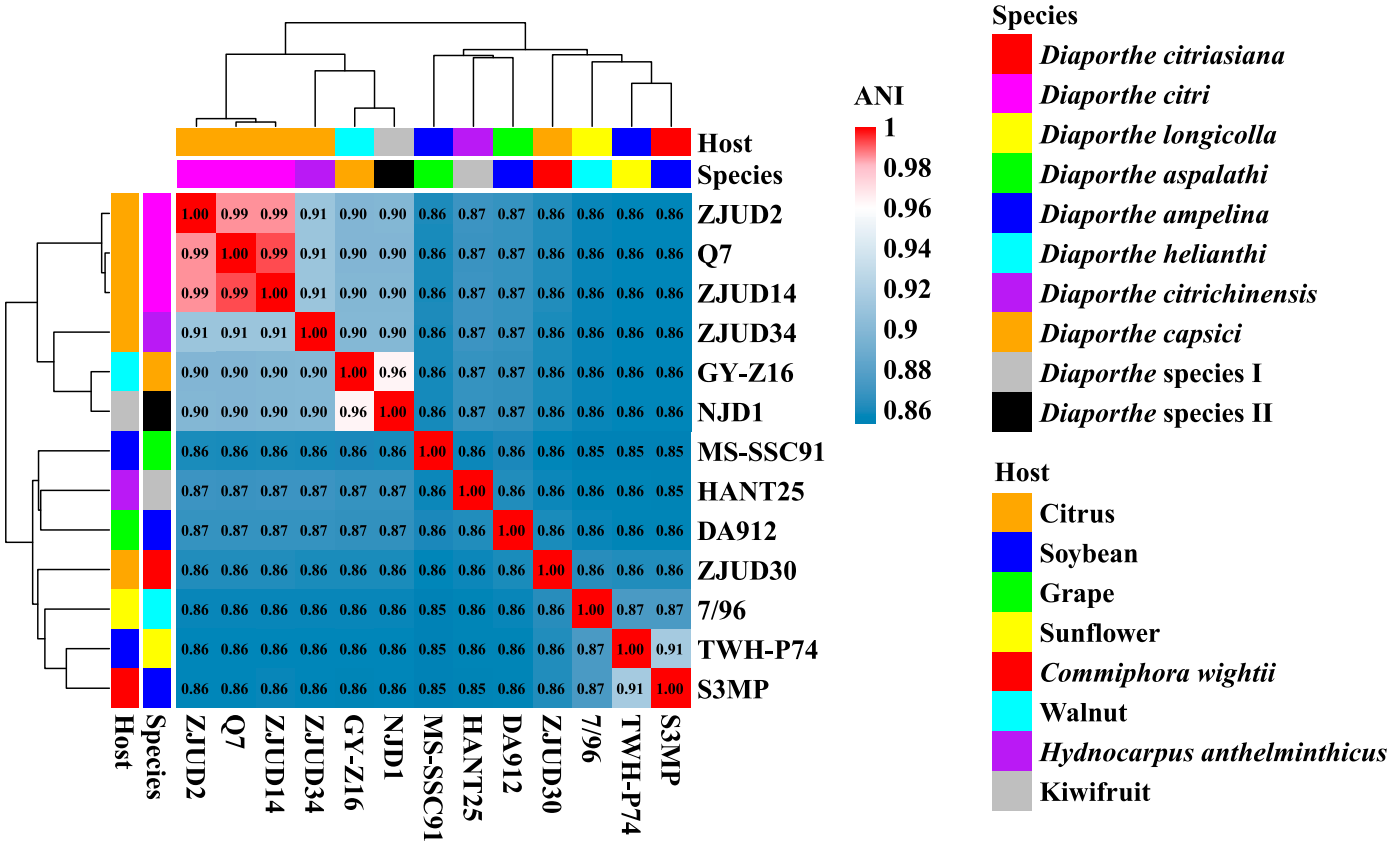

Fig. 1. The average nucleotide identity analyses of 13 *Diaporthe* strains shows that all three *D. citri* ZJUD2, ZJUD14, and Q7 have more than 99% nucleotide identity.

parameters of antiSMASH were with "-c 24–taxon fungi–genefinding-tool none–cf-create-clusters–clusterhmmer–cb-general–cb-subclusters–cb-knownclusters". Totally, the antiSMASH identified 98 to 102 SM clusters in *D. citri*, 88 SM clusters in *D. citriasiana* ZJUD30, and 110 SM clusters in *D. citrichinensis* ZJUD34 (Table 2).

For comparative genome sequence analysis, the genomes of *D. longicolla* strain TWH P74 (GenBank accession number JUJX00000000), *D. aspalathi* MS-SSC91 (LJJS00000000), *D. ampelina* S3MP (LWAD00000000) and DA912 (LCUC00000000), *D. helianthi* 7/96 (MAVT00000000), *D. capsici* GY-Z16 (WNXA00000000), *D. phragmitis* NJD1 (JACDXY000000000), and *Diaporthe* sp. HANT25 (JACBFG000000000) were downloaded from the GenBank database of the NCBI website (https://www.ncbi.nlm.nih.gov/nuccore). The average nucleotide identity (ANI) of different *Diaporthe* species was calculated using PYANI v0.2.10 (https://github.com/widdowquinn/pyani) and visualized using the R pheatmap package. The ANI heatmap showed that the ANI of all three *D. citri* strains ZJUD2, ZJUD14, and Q7 exceeded 99% (Fig. 1). Interestingly, we found that the ANI between *D. citri* and *D. citrichinensis* was 91%, indicating that there is a close genetic relationship between *D. citri* and *D. citrichinensis*. Then, we used OrthoFinder v2.2.7 combined with an all-versus-all protein BLAST to cluster the protein orthologous groups among different *Diaporthe* species (Emms and Kelly 2019). The core genes within the genus *Diaporthe* were visualized using the R UpSetR (Conway et al. 2017) and venn (https://cran.r-project.org/web/packages/venn/index.html) package. Protein homology analysis revealed that these seven *Diaporthe* species included 8,882 homologous groups, which constituted the core gene set of *Diaporthe* (Fig. 2). In conclusion, the *Diaporthe* genomes and the *Diaporthe* genomes database described in this paper are useful resources for citrus-related *Diaporthe* species, and can be used as reference genomes for analysis of the fungal biology, pathogen–host interaction, molecular diagnostic marker development, and population genomic analyses of citrus-related *Diaporthe* species in future studies.

Data availability statement. All the data of *Diaporthe* species reported here have been deposited at NCBI under BioProject accession number PRJNA660209. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession JADAZQ000000000 for *D. citri* strain ZJUD2, JADAZP000000000 for strain ZJUD14, and JADAZO000000000 for strain Q7. The assembled genome of *D. citriasiana* strain ZJUD30 has been deposited in GenBank under accession number JADAZS000000000. The assembled genome of *D. citrichinensis* strain ZJUD34 has been deposited in GenBank under accession number JADAZR000000000. The Illumina Hiseq raw reads have been deposited in the Sequence Read Archive (SRA) database of NCBI under accession number SRR12578329 for *D. citri* strain ZJUD2, SRR12578328 for ZJUD14, SRR12578327 for Q7, SRR12883652 for

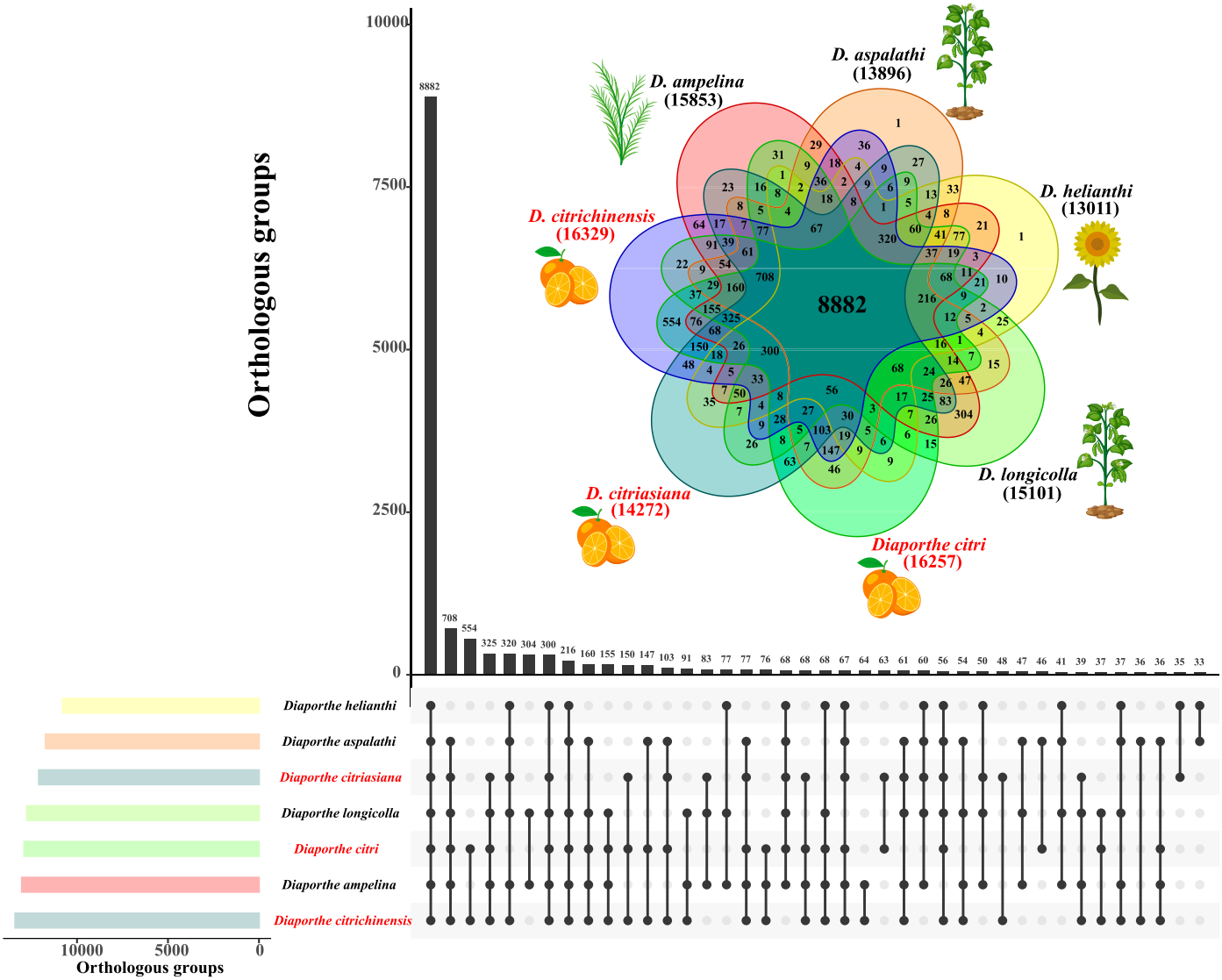

Fig. 2. Numbers of orthologous groups that are unique to each isolate, specific to two or more isolates, and common to all *Diaporthe* isolates.

D. citriasiana ZJUD30, and SRR12883651 for *D. citrichinensis* ZJUD34. All genome assemblies and proteomes are available in the Zenodo repository: https://zenodo.org/record/4091003. The figures, R codes, and custom Perl scripts are available on the figshare repository: https://doi.org/10.6084/m9.figshare.13095335. All the genome assemblies and annotation data generated in this study have been deposited on the citrus-related *Diaporthe* genome database at zjudata.com/blast/diaporthe.php.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Citrus-Related Diaporthe Database: http://www.zjudata.com/blast/diaporthe.php
SignalP-5.0 Server: http://www.cbs.dtu.dk/services/SignalP/
Pathogen–Host Interaction (PHI) database: http://www.phi-base.org/
antiSMASH fungal version: https://fungismash.secondarymetabolites.org/
Carbohydrate-Active enZYmes Database: http://www.cazy.org/
Benchmarking Universal Single-Copy Orthologs (BUSCO): https://busco.ezlab.org/

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