The "Bipartite" Structure of the First Genome of *Ampelomyces quisqualis*, a Common Hyperparasite and Biocontrol Agent of Powdery Mildews, May Point to Its Evolutionary Origin from Plant Pathogenic Fungi

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Abstract

Powdery mildews are among the most important plant pathogens worldwide, which are often attacked in the field by mycoparasitic fungi belonging to the genus *Ampelomyces*. The taxonomy of the genus *Ampelomyces* is unresolved, but well-supported molecular operational taxonomic units were repeatedly defined suggesting that the genus may include at least four to seven species. Some *Ampelomyces* strains were commercialized as biocontrol agents of crop pathogenic powdery mildews. However, the genomic mechanisms underlying their mycoparasitism are still poorly understood. To date, the draft genome of a single *Ampelomyces* strain, designated as HMLAC 05119, has been released. We report a high-quality, annotated hybrid draft genome assembly of *A. quisqualis* strain BRIP 72107, which, based on phylogenetic analyses, is not conspecific with HMLAC 05119. The constructed genome is 40.38 Mb in size, consisting of 24 scaffolds with an N50 of 2.99 Mb and 96.2% completeness. Our analyses revealed "bipartite" structure of *Ampelomyces* genomes, where GC-balanced genomic regions are interspersed by longer or shorter stretches of AT-rich regions. This is also a hallmark of many plant pathogenic fungi and provides further evidence for evolutionary affinity of *Ampelomyces* species to plant pathogenic fungi. The high-quality genome and annotation produced here provide an important resource for future genomic studies of mycoparasitisim to decipher molecular mechanisms underlying biocontrol processes and natural tritrophic interactions.

Key words: mycoparasite, Dothideomycetes, genomic resources.

Significance

Mycoparasitic fungi belonging to the genus *Ampelomyces* attack powdery mildews, which are important plant pathogenic fungi worldwide. Despite their ecological significance, and importance as biocontrol agents of crop pathogenic powdery mildews, limited genetic resources are available for *Ampelomyces*. The first high-quality assembly and annotation of the *A. quisqualis* genome produced here will provide an invaluable resource for genomic studies of mycoparasitism and points to possible evolutionary origin of *Ampelomyces* from plant pathogenic fungi.

Introduction

Powdery mildews (Erysiphaceae), common obligate biotrophic plant pathogens, are often attacked in the field by mycoparasitic fungi belonging to the genus *Ampelomyces*. As powdery mildews are themselves parasites, *Ampelomyces* spp. are also considered as hyperparasites (Kiss 2001; Parratt and Laine 2016). The natural tritrophic interactions between host plants, powdery mildews, and *Ampelomyces*

spp. have been intensively studied in the field from an eco-logical context (Kiss 2008; Kiss et al. 2011; Tollenaere et al. 2014; Pintye et al. 2015; Numminen et al. 2019). Some *Ampelomyces* strains have been commercialized as biocontrol agents of crop pathogenic powdery mildews (Boddy 2015; Legler et al. 2016). The taxonomy of the genus *Ampelomyces* is unresolved, but well-supported molecular operational tax-onomic units (MOTUs) were defined suggesting that the ge-nus may include at least four to seven species (Liang et al. 2007; Park et al. 2010; Kiss et al. 2011; Angeli et al. 2012; Pintye et al. 2012; Liyanage et al. 2018; Németh et al. 2019; Németh, Mizuno, et al. 2021). All strains belonging to the genus *Ampelomyces* are hyperparasites of powdery mildews just like the type species *A. quisqualis*. The name *A. quisqualis* has been applied to phylogenetically diverse *Ampelomyces* hyperparasites belonging to different MOTUs (Park et al. 2010; Angeli et al. 2012; Liyanage et al. 2018), which high-lights the need for a taxonomic reassessment of this binomial (Kiss et al. 2004; Legler et al. 2016). Experiments with *Ampelomyces* transformants exhibiting the green fluorescent protein (GFP) indicated that these hyperparasites cannot thrive as saprobes; living powdery mildew colonies constitute their primary niche (Németh et al. 2019).

The GFP study has established a framework for a molecular genetic toolbox for *Ampelomyces* strains (Németh et al. 2019), which was later applied in a gene knock-out project (Németh, Li, et al. 2021). In terms of genomic resources, these hyperparasites are largely unexplored: to date, the draft ge-nome of a single *Ampelomyces* strain, designated as HMLAC 05119, has been released (Haridas et al. 2020). The strain was isolated from an undetermined powdery mildew infecting *Youngia japonica* in China (Liang C, personal communica-tion). The transcriptome of another *Ampelomyces* strain, CNCM I-807 or M-10, which was commercialized as the ac-tive ingredient of the AQ10 Biofungicide product in the United States and the European Union (Legler et al. 2016), is the only other genomic resource available to date for the genus *Ampelomyces*. The AQ10 strain was isolated from an undetermined powdery mildew infecting *Catha edulis* in Israel (Legler et al. 2016). The analysis of its transcriptome during the early and the late stages of parasitism revealed the upre-gulation of some genes related to toxin biosynthesis, together with other potentially mycoparasitism-related proteins such as secreted proteases and putative virulence factors during mycoparasitism (Siozios et al. 2015). However, approximately 50% of the *Ampelomyces* transcripts did not point to any known protein sequences (Siozios et al. 2015). This may indi-cate that a part of the *Ampelomyces* proteome is unique, or this type of mycoparasitism has not been studied in sufficient detail in other interfungal parasitic relationships.

Here, we present a high-quality, annotated hybrid draft genome assembly of *A. quisqualis* strain BRIP 72107. Our phylogenetic analysis presented here revealed that BRIP 72107 is not conspecific with HMLAC 05119, the only

Ampelomyces strain with a known genome (Haridas et al. 2020). BRIP 72107 is, however, conspecific with the commer-cial AQ10 strain, and both belong to the MOTU that has included many *Ampelomyces* strains newly isolated from the field in diverse studies in China, Europe, Japan, the United States, and Korea (Liang et al. 2007; Park et al. 2010; Kiss et al. 2011; Pintye et al. 2012; Liyanage et al. 2018; Németh, Mizuno, et al. 2021). Therefore, this genome will be useful to decipher molecular mechanisms underlying both biocontrol processes and natural tritrophic interactions.

Results and Discussion

Genome Assembly and Annotation

Ampelomyces quisqualis strain BRIP 72107 was assembled into 24 scaffolds with a total assembly size of 40,378,121 bp and genome completeness of 96.2% (table 1). Of the total of 3,786 Dothideomycetes Benchmarking Universal Single-Copy Orthologs (BUSCOs) searched, BRIP 72107 included 3,641 complete (96.2%), 16 fragmented (0.4%), and 129 missing (3.4%) BUSCOs. Based on a genome size of 42 Mb estimated by Jellyfish, and a total of 8.8 and 8 Gb of sequence data generated by MinION and Illumina MiSeq platforms, respectively, we estimate a genome cover-age of 400×. A combination of ab initio and evidence-based gene modeling with two additional rounds of gene predic-tions after training SNAP in Maker pipeline resulted in 22,470 predicted exons within 10,439 genes (including 7,255 evidence-supported gene models), including 4,203 with 3'-UTRs and 4,345 with 5'-UTRs.

Bipartite Genome of *A. quisqualis*

The genome of BRIP 72107 has a bimodal GC content, due to the presence of gene-rich, GC-balanced regions interspersed by long or short stretches of AT-rich, gene-sparse regions (fig. 1). This phenomenon was observed in almost all the assem-bled contigs, with AT-rich regions varying in size from 15 to 270 kb. The "bipartite" feature of the genome is also a hall-mark of many plant pathogenic fungi that is hypothesized to arise when duplicated DNA, such as transposons, undergoes C to T transitions by the process of repeat-induced point mu-tation (Testa et al. 2016). This substantial proportion of repet-itive and AT-rich regions has been proposed to result in the "two-speed" evolution of these genomes, where genes lo-cated close to the AT-rich regions (mostly secreted proteins) have higher rates of evolution (Dong et al. 2015). Recent analyses revealed a surprisingly close phylogenetic relationship between *Ampelomyces* mycoparasites and plant pathogens such as *Parastagonospora nodorum* and *Leptosphaeria mac-ulans* (Haridas et al. 2020), and the *Ampelomyces* genus is classified in the *Leptosphaeriaceae* family that includes many plant pathogens and other plant-associated species. These results, together with the bipartite genome structures of all

Table 1

Genome Statistics for *Ampelomyces* Strains Sequenced to Date

Strain[a]	Powdery Mildew Host	Plant Host	Assembly Size (Mb)	Cov[b]	No. of Contigs	Contig N50 (bp)	No. of Scaffolds	Scaffold N50 (bp)	No. of Ns per Mb	GC Content (%)	Genome Completeness (%)[c]	NCBI Accession Number
BRIP 72107	*Golovinomyces bolayi*	*Cestrum parqui*	40.38	400×	25	2,994,887	24	2,994,887	2	45.4	96.2	JAGTXZ000000000
HMLAC 05119	Undetermined powdery mildew	*Youngia japonica*	36.81	103×	468	258,565	73	4,300,649	9,771	46.4	96.3	VOSX00000000.1

[a]Strain BRIP 72107 was sequenced in the current study. HMLAC 05119 was obtained from the JGI Genome Portal (Haridas et al. 2020).
[b]Genome coverage.
[c]Genome completeness for the two genomes was determined based on benchmarking universal single-copy orthologs (BUSCOs) (Simão et al. 2015) against the dothideomycetes_odb10 database.

these fungi, may point to the evolutionary origin of *Ampelomyces* from plant pathogenic fungi.

Phylogenetic Analysis

Phylogenetic analysis of 28 *Ampelomyces* strains based on the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA), including the 5.8S rRNA gene, revealed a high level of diversity within the genus *Ampelomyces*, with nine well-supported MOTUs (fig. 1E), which highlighted the need for a taxonomic reassessment of the genus *Ampelomyces*. Strain BRIP 72107 belonged to the strongly supported clade/MOTU 1, together with the commercial strain AQ10, whereas HMLAC 05119 was part of MOTU 4. This indicated that the two strains are not conspecific. Another strain, 94013, commercialized as Q-fect in Korea (Park et al. 2010), also belonged to MOTU 4. Previous phylogenetic analyses of *Ampelomyces* strains also concluded that the genus consists of multiple species (Liang et al. 2007; Park et al. 2010; Kiss et al. 2011; Angeli et al. 2012; Pintye et al. 2012; Liyanage et al. 2018).

Materials and Methods

Sample Collection and Culturing

Strain BRIP 72107 was isolated from *Golovinomyces bolayi* infecting *Cestrum parqui* collected in Toowoomba, Queensland, Australia. The strain is available from the Queensland Plant Pathology Herbarium (BRIP), which includes a large collection of living fungal and bacterial strains, in addition to herbarium specimens. The isolation process was done as described by Liang et al. (2007). The identity of strain BRIP 72107 as *Ampelomyces* was confirmed via sequencing the nrDNA ITS region using universal primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Four-week-old mycelia of BRIP 72107 grown in potato dextrose broth were lyophilized overnight. One hundred milligrams of lyophilized mycelia were flash-frozen in liquid nitrogen and ground with stainless steel beads (2.8 mm diameter; Sigma–Aldrich) in a FastPrep-24 (MP Biomedicals, Australia) at 6.5 m/s for 30 s and stored at −80 °C until DNA extraction.

DNA and RNA Extraction

For long-read sequencing, high-molecular weight (HMW) DNA was extracted using a chloroform/isoamyl alcohol extraction method with an isopropanol precipitation as described by Feehan et al. (2017). Briefly, ground mycelia were lysed in 700 µl lysis buffer (potassium metabisulfite 0.25 M, Tris 0.2 M pH 7.5, ethylenediaminetetraacetic acid 50 mM, NaCl 2 M, 2% CTAB, ddH$_2$O) pre warmed to 65 °C with 300 µl 5% Sarcosyl prewarmed to 65 °C and incubated for 30 min at 65 °C. DNA was isolated by chloroform/isoamyl alcohol extraction and subsequent isopropanol precipitation. RNase

FIG. 1.—The first 1,400,000 bp of BRIP 72107 contig-4 is demonstrated here as an example showing GC-balanced regions interspersed by longer or shorter stretches of AT-rich regions (A). The GC content distribution of *Ampelomyces* spp. (*B* and *C*) show striking similarity to the plant pathogenic fungus, *Leptosphaeria maculans* (*D*). Vertical blue lines show the GC cut-off points selected by OcculterCut (Testa et al. 2016) to classify genome segments into

(10 mg/ml) treatment was performed for 2 h at 37 °C and finally the DNA was cleaned with AMPure XP beads (Beckman Coulter). HMW DNA was purified using the Qiagen Genomic-tip 20/G kit according to manufacturer's instructions and quantified using the Qubit v.3.0 fluorometer (ThermoFisher Scientific, Australia). Quality ratios were checked using the Denovix DS-11 series (Life Science Technologies) and the integrity was assessed through electrophoresis on a 0.8% agarose gel containing 1:20,000 GelRed (Biotium, Australia), then stored at −20 °C. For Illumina MiSeq sequencing, DNA was extracted from lyophilized fungal mycelia using a DNeasy Plant Mini Kit (Qiagen, Australia) according to manufacturer's instructions, except for the final step where DNA was eluted in 10 mM filter-sterilized Tris–HCl (pH 8.5).

Total RNA from fresh fungal mycelia flash-frozen and ground in liquid nitrogen was extracted using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions using the purification of total RNA from plant cells and tissue and filamentous fungi. The final product was checked via agarose gel electrophoresis and quantified using a Qubit v.3.0 fluorometer (ThermoFisher Scientific, Australia) and submitted to the Australian Genome Research Facility (Melbourne, Australia) for total mRNA sequencing.

Genome Sequencing

Long-read sequencing was performed using Oxford Nanopore Technology (ONT). A MinION library was constructed from 1,000 ng DNA using a Genomic DNA by Ligation kit (SQK-LSK109; ONT, Oxford, United Kingdom) according to the standard protocol. The library was loaded onto a MinION FLO-MIN 106 R9.4.1 flow cell and sequenced for 39 h. Read quality statistics were assessed using Nanoplot v.1.28.2 on Galaxy Australia Portal (Afgan et al. 2018). For Illumina short-read sequencing, library preparation was conducted on 150 ng DNA using an Illumina DNA Prep kit and Nextera DNA CD Indexes (Illumina, Singapore) according to manufacturer's instructions. The library was sequenced on an Illumina MiSeq platform using a 600-cycle paired-end V3 reagents kit. Read quality statistics were assessed using FastQC v.0.11.8 (Andrews 2010) on Galaxy Australia Portal.

Read Preparation, Genome Assembly, and Annotation

All raw data were screened and filtered for bacterial contamination using Kraken v.2.1.1 (Wood and Salzberg 2014).

Adapter removal from Illumina reads was conducted using BBduk from the BBmap suite v.36.86 (Bushnell 2014). The Kmer counting software Jellyfish v.2.3.0 (Marçais and Kingsford 2011) was implemented to estimate the genome size using the Illumina reads. Porechop v.0.2.4 (Wick 2017) was used for adapter removal from the raw MinION reads. The hybrid assembler MaSuRCA v.3.3.3 (Zimin et al. 2013) was used. Raw Illumina reads without barcode-removal and quality filtering were used for MaSuRCA assembly as recommended by the developer. The completeness of the genome assembly was evaluated via Benchmarking Universal Single-Copy Orthologs (BUSCO) v.1.2 (Simão et al. 2015). Genome statistics of the generated assembly were compared with that of HMLAC 05119 (Haridas et al. 2020) using Quast v.2.0.5 (Gurevich et al. 2013). The program OcculterCut v.1.1 (Testa et al. 2016) was used to scan the genome of *A. quisqualis* strain BRIP 72107, *Ampelomyces* sp. strain HMLAC 05119 (Haridas et al. 2020), and *Leptosphaeria maculans* strain v.23.1.3 (Rouxel et al. 2011) to determine their percent GC content distribution.

Transcriptome assembly was conducted using Trinity v.2.10.0 (Grabherr et al. 2011). Genome annotation was conducted using Maker v.2.31.9 (Cantarel et al. 2008). A repeat library was generated with RepeatModeler v.2.0.1 (Smit and Hubley 2008) and repeats were masked prior to annotation. A first round of RNA-evidenced gene prediction was conducted using Maker. The resulting annotation was used to produce a hidden Markov model (HMM) profile for *A. quisqualis*, which was further refined with a second round of SNAP training and used for the final annotation (Cantarel et al. 2008).

Phylogenetics Analyses

To depict the molecular diversity within the genus *Ampelomyces* and phylogenetic relationship of strains BRIP 72107 and HMLAC 05119, a Bayesian phylogram was constructed using MrBayes v.3.2.4 (Ronquist et al. 2012) based on the GTR+I+G nucleotide substitution model selected using PAUP v.4.0b10 (Swofford and Sullivan 2003) and MrModeltest v.2.3. (Nylander 2004). ITS sequences of reference isolates were obtained from NCBI GenBank database. An ITS sequence was not available for strain HMLAC 05119; therefore, ITS sequence of BRIP 72107 was used as a query against its published genome (GenBank accession number: VOSX00000000.1) to extract the ITS region of HMLAC 05119 and include in the phylogenetic analysis.

distinct AT-rich and GC-balanced regions. The percentage values shown on the left and right sides of the vertical blue lines indicate the percentage of the genome classified as AT-rich and GC-balanced, respectively. (*E*) The majority rule consensus Bayesian phylogram inferred from the internal transcribed spacer sequences of the nuclear ribosomal DNA and the intervening 5.8S region (TreeBASE no. 28185). Tip labels include *Ampelomyces* strain number, the powdery mildew host, the plant host, and the country of origin. NCBI GenBank accession numbers used to construct the tree are provided in parentheses. The tip labels in bold represent *Ampelomyces* strains with available whole genome assemblies: BRIP 72107 sequenced in the current study and HMLAC 05119 (Haridas et al. 2020). Bayesian posterior probability values are shown at the branches. The tree is rooted to *Phoma herbarum* strain CBS 276.37. The scale bar represents nucleotide substitutions per site.

Acknowledgments

Data Availability

This Whole Genome Shotgun project has been deposited at GenBank under the accession number JAGTXZ000000000. The version described in this article is version JAGTXZ010000000. The alignment and phylogenetic tree are deposited in TreeBASE (No. 28185).

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