



Draft Genome Sequencing of *Rhizoctonia solani* Anastomosis Group 3 (AG3- PT) Causing Stem Canker and Black Scurf of Potato

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Abstract *Rhizoctonia solani* is a soil-borne basidiomycete fungus with a necrotrophic lifestyle being classified into fourteen reproductively incompatible anastomosis groups (AGs). AG3-PT (a potato subgroup) is associated with quantitative and qualitative yield losses through stem canker and black scurf in potato. Here we present the first draft sequence of the *R. solani* [AG3-PT] strain RS-20 with a G-C content of 48.3%. It consists of 11,431 total predicted protein coding regions including 181 tRNA and 31 rRNA coding genes. The initial pBLAST revealed more than 97% hits among AG groups where as only 1.7% of genes hit with other organisms. The *R. solani* genome is found to be dominated with tri mer repeats. The genome-wide evolutionary studies revealed the close association of AG3-PT with AG3. The draft sequence represents a helpful resource not only for understanding the core genes involved in pathogenecity but also evolution and adaptive behaviour within the *R. solani* species complex.

Resumen *Rhizoctonia solani* es un hongo basidiomiceto del suelo con un estilo de vida necrotrófico que se clasifica dentro de los 14 grupos de anastomosis (AGs) de incompatibilidad reproductiva. AG3-PT (un subgrupo de papa) esta asociado con pérdidas de rendimiento cuantitativas y cualitativas por vía de cáncer de tallo y por costra negra en papa. Aquí presentamos la primera secuencia en borrador del *R. solani*

[AG3-PT] variante RS-20 con un contenido de G-C de 48.3%. Consiste de 11,431 regiones totales de codificación predicha de proteínas incluyendo 181 tARN y 31 rARN genes de codificación. El pBLAST inicial reveló más del 97% de coincidencias entre los grupos AG, en donde tan solo el 1.7% de los genes coincidieron con otros organismos. El genomio de *R. solani* se encuentra dominado con repeticiones de trimeros. Los estudios de la evolución de la amplitud del genoma revelaron la asociación cercana de AG3-PT con AG3. La secuencia preliminar representa un recurso útil, no solo para entender los genes básicos involucrados en la patogenicidad, sino también la evolución y el comportamiento adaptativo dentro del complejo de la especie *R. solani*.

Keywords Species complex · Potato type · Pathogenicity · AUGUSTUS · Repeats · Phylogeny

Abbreviations

RS-20	<i>Rhizoctonia solani</i> Strain 20
AG	Anastomosis Groups
PT	Potato type
NaOCl	Sodium Hypochlorite
PDA	Potato Dextrose Agar
CTAB	Cetyi Trimethylammonium bromide
ITS	Internal Transcribed Spacer
CDS	Coding DNA Sequence
Mb	Mega base (10 ⁶ base)
Kb	Kilo base (10 ³ base)
MFS	Major Facilitator Superfamily
MISA	MIcroSAteLLite
ANI	Average Nucleotide Identity
PTP	Peco Titre Plate

Type culture is available with Dr. Vinay Sagar

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Introduction

The fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* ([A. B. Frank] Donk) (*Deuteromyces*, *Mycelia Sterilia*) is responsible for many economically important plant diseases worldwide and also synonymed as *Rhizoctonia praticola*. The fungus can also degrade organic matter in soil as a saprobe and represents an important evolutionary link between beneficial and plant disease-causing fungi (Cubeta and Vilgalys 2000). It is divided into subgroups called anastomosis groups (AGs), in which isolates are categorized according to the ability of their hyphae to anastomose (fuse) with one another. At present, the *R. solani* species complex is comprised of fourteen anastomosis groups (AGs), most of which are reproductively incompatible with each other and are numbered AG1 through AG13 (Sneh et al. 1991). The ‘bridging isolate’ AG-BI is the exception, being compatible with multiple AGs (Gracia et al. 2006). It has been established that all AGs and their sub-groups are genetically isolated and represent highly divergent evolutionary units (Rosewich et al. 1999; Ceresini et al. 2007).

Anastomosis group 3 (AG3) is a common inhabitant of the soil ecosystem and has a worldwide distribution. It is an economically important pathogen that affects food crops mostly in the plant family *Solanaceae* including eggplant, pepper, tomato and potato. *R. solani* AG3-PT (The potato type) is a subgroup of AG3 and main causal agent of stem, stolon cancer and black scurf of potato world wide including India. It is both tuber and soil borne. Loss of quantity and in particular quality caused by black scurf disease reaches even up to 50% (Keiser 2008; Woodhall et al. 2013). Although, many disease management strategies have been developed like chemical spray, maintaining low soil pH, avoiding areas with history of disease or long crop rotation, use of disease free certified seeds, seed treatment, timely harvest, trichoderma and other biological treatments, but so far none of these have proven to be completely effective (Khaldi et al. 2015). To date genomes of strains belonging to only four AGs have been sequenced viz. AG1 [AG1-IA (Zheng et al. 2013), AG1-IB (Wibberg et al. 2013)], AG2-IIIB (Wibberg et al. 2015b), AG3 (Cubeta et al. 2014) and AG8 (Hane et al. 2014). In order to gain insight into the infection and pathogenesis processes, the complete genome of AG3-PT strain RS-20 was sequenced to understand the mechanism of pathogenicity as well as to determine its evolutionary relationship with other *Rhizoctonia* species complex.

Material and Methods

Freshly harvested tubers of cv. Kufri Jawahar in the rabi season of 2010 from Kufri, (Himachal Pradesh, India; 31.0979°N 77.2678°E, located in mid himalya and having wet temperate

climate) infected with black scurf were collected. The tuber samples were surface sterilized with 1% NaOCl; sections of affected tissue were excised and plated onto potato dextrose agar (PDA) media. *Rhizoctonia*-like colonies were identified and the pathogen was purified after repeated sub-culturing on PDA. Fungal mycelia was harvested from 10 to 15 days old culture broth. Genomic DNA was isolated from harvested mycelia using CTAB (Murray and Thompson 1980) method and its identity confirmed by comparing ITS (ITS1-F and ITS4 Primers) sequences with AG3-PT sequences from GenBank (JX27814 and KC157664) (Fiers et al. 2010). The purified DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher, India) and used for genomic rapid library preparation (Roche Diagnostics) using the kits and user guide provided. Further sequencing was done using Roche 454 (GS-FLX Titanium) pyrosequencing platform. Two shotgun sequencing runs were carried out with complete region of PTP (Peco Titre Plate). The raw sequence data was assembled using GS *DeNovo* Assembler (Version 2.5.3) and GS Reference Mapper (Roche Diagnostics). Protein coding regions were predicted using eukaryotic GeneMark.hmm (Version 2.2a) and AUGUSTUS (<http://bioinf.uni-greifswald.de/augustus/submission/>) for cross validation. The data was also used for getting the average number of exons per gene manually. Open software RNAmmer 1.2 Server (www.cbs.dtu.dk/Services/RNAmmer/) and FindtRNA Version 1.0 (www.bioinformatics.org/findtrna/FindtRNA.html) were used for finding the total number of rRNA and tRNA coding genes respectively. Genome-wide repeats were studied using MicroSATellite identification tool ([MISA-http://pgrc.ipk-gatersleben.de/misa/](http://misa-ipk-gatersleben.de/misa/)) and evolutionary relationship among the *R. solani* Anastomosis Groups (AGs) was analysed by Average Nucleotide Identity (ANI) using one-way ANI among *R. solani* Anastomosis Groups (AGs) datasets, as calculated by (Goris et al. 2007) (<http://enve-omics.ce.gatech.edu/ani/>).

Results and Discussion

The shot gun sequences yielded high quality 2,827,025 reads amounting to 1,031,371,409 bases which was equivalent to 17-fold coverage of its estimated ≈ 60 Mb genome. The GS *De Novo* Assembler (version 2.5.3) using the heterozygotic mode yielded 21,475 contigs (over 500 bp) with an average contig size of 4.1 kb, N50 contig size of 4068 bp and largest contig of 97.2 kb. The proportion of bases called that had a quality score of 40 or above was 98.66%. The contigs covered more than 54.2 Mb genome size of the pathogen. The draft genome of AG3-PT strain RS-20 has G-C content of 48.3%, which is comparable to all the other anastomosis groups sequenced so far that varies between 48.1 to 48.8%, except for the AG1-IA having GC content of 47.61%

Table 1 List of sequenced *Rhizactonia solani* and their genome characters

SN	<i>R. solani</i> -AG	Strain	Host	Seqd Size (Mb) ^a	Contigs reported ^b	GC Content (%) ^c	Genes Predicted ^d	Gene Density (genes/10 Kbp)	tRNA ^e	rRNA ^f	Reference
1	AG1-IA	B275	Rice	37.80	2648	47.61	10,489	2.8	153	18	Zheng et al. 2013
2	AG1-IB	7/3/14	Soybean	52.74	23,355	48.10	12,616	2.4	187	22	Wibberg et al. 2013 Wibberg et al. 2015a
3	AG2 IIIB	BBA69670	Sugar Beet	56.02	5826	48.34	11,897	2.1	135	15	Wibberg et al. 2015b
4	AG3	Rhs-1AP	Potato	52.50	6040	48.40	12,726	2.4	-	17	Cubeta et al. 2014
5	AG3-PT	RS-20	Potato	55.85	30,594	48.30	11,431	2.1	181	31	This study
6	AG8	WAC10335	Lupin	35.92	-	48.80	13,964	3.9	105	20	Hane et al. 2014

^aThe estimated genome size in Mega bases

^bFigures taken from all *R. solani* Bio projects submitted to NCBI/Genebank

^cGC% content of the genome as estimated from CLC Bio V. 7.0

^dPredicted number of genes common from both eukaryotic GeneMark.hmm (Version 2.2a) and AUGUSTUS,

^eTotal tRNA coding genes as predicted by FindtRNA Version 1.0

^fTotal rRNA coding genes as predicted by RNAmmer 1.2 Server

(Wibberg et al. 2013; Wibberg et al. 2015a). Gene prediction using the eukaryotic GeneMark.hmm (Version 2.2a) and AUGUSTUS revealed a total of 11,431 protein coding regions (CDSs) common in both, spread over genome covering 13.36 Mb (29.4% of genome as coding). This number is very close to the predicted genes number of *AG1-IA* strain B275 (10,489) (Zheng et al. 2013), *AG1-IB* strain 7/3/14 (12,616) (Wibberg et al. 2013; Wibberg et al. 2015a), *AG2-IIIB* strain BBA69670 (11,897) (Wibberg et al. 2015b), *AG3*, strain *Rhs-1AP* (12,726) (Cubeta et al. 2014) and *AG8* strain WAC10335 (13,964) (Hane et al. 2014). The average exons per gene was found to be 5.24 and the average gene density was calculated to be 2.08 per 10 Kb. Total rRNA genes predicted to be 31 using RNAmmer 1.2 Server for eukaryotic genome (Lagesen et al. 2007) and the total tRNA coding genes were predicted to be 181 using FindtRNA Version 1.0 (Table 1). The initial pBLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) of predicted genes against the non redundant protein sequences ($e \leq 10^{-9}$) revealed that the distribution of the predicted proteins among functional

classes was similar to that of *R. solani* viz. 43.5% with *R. solani* AG3 (strain *Rhs-1AP*), 39.3% with *R. solani* (Strain 123E), \approx 2.5% with both *R. solani* AG8 and AG1-IB and Nearly 1.7% of the genes had hits with other organisms and in general hits were dominated by membrane transporter MFS 1 (Major Facilitator Superfamily).

The repeat region studies using MicroSatellite identification tool revealed abundance of mono, di and tri mer repeats in the genome. Number of hexa mer repeats found to be higher than tetra and penta mers, and this feature was found across the genomes of anastomosis groups (AGs). A total of 178 predicted gene sequences had at least one microsatellite in them (genic microsatellites) and only 11 genes possessed more than one microsatellites. Tri-mer repeats dominated the genic microsatellites (80%) and only 11% of these are in the compound form. When compared to the *Rhizoctonia* genomes, *AG1-IB* had the highest number of microsatellites in compound form whereas AG8 had the least. The genome-wide microsatellite information can be handy for diversity and classification studies.

Table 2 Average Nucleotide Identity using one-way ANI among *Rhizactonia solani* Anastomosis Groups (AGs) and selected fungi from class Agaricomycetes datasets*, as calculated by Goris et al. 2007

Anastomosis Group	Strain	1	2	3	4	5	6	7	8	9
1	AG3-PT	RS-20	100							
2	AG3	Rhs-1AP	97.75	100						
3	AG8	WAC10335	81.73	81.53	100					
4	AG2-IIIB	BBA69670	80.03	80.21	79.97	100				
5	AG1-IB	7/3/14	76.09	76.02	75.89	75.82	100			
6	AG1-IA	B275	75.87	75.78	75.92	75.62	79.00	100		
7	<i>C. neoformans</i>	JEC21	82.33	82.15	71.88	82.98	87.75	84.46	100	
8	<i>C. cinerea</i>	Okayama 7#130	77.40	76.20	75.44	75.39	76.96	76.69	79.12	100
9	<i>P. indica</i>	DSM11827	75.02	75.63	72.55	74.98	76.68	74.20	71.67	75.38

*The complete or partial gene sequences of around 100 housekeeping genes which include A) Ribosomal genes, B) Cytochrome genes C) Translation elongation factors (TEFs), D) ATPases, E) Translation release factors (eRFs), F) Alcohol dehydrogenase genes, G) Chitin related genes H) DNA and RNA polymerase genes I) Histone related genes, J) ABC transporter genes, K) major facilitator superfamily (MFS) genes

Average Nucleotide Identity (ANI) using one-way ANI among *R. solani* Anastomosis Groups (AGs) datasets, as calculated by (Goris et al. 2007), revealed a close association between AG3-PT (Strain RS-20) and AG3 (Strain Rhs-1AP) (97.75%) re-confirming its recent divergence from the AG3 based on the environment and host adaptation, whereas, least association was found with AG1-IA (Strain B275) (75.87%). The codon preference studies revealed that Methionine (ATG) and Tryptophan (TGG) have single codon coding for respective amino acids and TGA (43.2%) is the preferred stop codon over TAG (27.5%) and TAA (29.2%). The codon preference was found to exist for all the amino acids ranging from 3% to 15% over the non-preferred. The ANI with *Coprinopsis cinerea* (Order: Agaricales), *Cryptococcus neoformans* (Order: Tremellales) and *Piriformospora indica* (Order: Sebaciales) belonging to class Agaricomycetes was found to be 77.40%, 82.33% and 75.02% respectively indicating the phylogenetically, greater closeness of order catharellales (*Rhizoctonia sp.*) with tremellales (Table 2). These preliminary studies of the genomes indicate the presence of greater diversity among the anastomosis groups of *R. solani* and could need further sub-classification among these orders. A detailed genomic study needs to be taken up among sequenced genomes of the class agaricomycetes for better understanding and to fine-tune their systemic classification.

There is necessary for more genomes representing all of the *R. solani* Anastomosis Groups to be sequenced, as enormous genome sequence data provide new opportunities for comparative genomics between *R. solani* anastomosis groups. This is the first draft genome sequence report for *R. solani* (AG3-PT) sub type and the availability of the genome sequence is certain to be a vital resource in epidemiological and quarantine studies. Validation of predicted genes would require additional transcriptome sequencing and expression experiments. A detailed examination of this genome and inter-comparison with other *Rhizoctonia* species complex genomes could expand our understanding of evolutionary relationships to obtain unexpected new insights into their origins, diversity, pathogenicity, and host-specificity. Further, functional characterization of potential effector genes is requirement for determining their roles in pathogenesis which needs to be carried out across anastomosis groups of *R. solani*.

Nucleotide Sequence Accession Numbers Sequences from this whole-genome shotgun project have been deposited at NCBI/GeneBank under Bioproject **PRJNA316175** and accession number **LVWR00000000**. The version described in this paper is the first version, **LVER01000000**. The strain is available with Division of Plant Protection, ICAR-CPRI, Shimla and also has been submitted to MTCC, CSIR-IMTECH, Chandigarh.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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