Development of a *Rhizoctonia solani* AG1-IB Specific Gene Model Enables Comparative Genome Analyses between Phytopathogenic *R. solani* AG1-IA, AG1-IB, AG3 and AG8 Isolates

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Abstract

*Rhizoctonia solani*, a soil-born plant pathogenic basidiomycetous fungus, affects various economically important agricultural and horticultural crops. The draft genome sequence for the *R. solani* AG1-IB isolate 7/3/14 as well as a corresponding transcriptome dataset (Expressed Sequence Tags—ESTs) were established previously. Development of a specific *R. solani* AG1-IB gene model based on GMAP transcript mapping within the eukaryotic gene prediction platform AUGUSTUS allowed detection of new genes and provided insights into the gene structure of this fungus. In total, 12,616 genes were recognized in the genome of the AG1-IB isolate. Analysis of predicted genes by means of different bioinformatics tools revealed new genes whose products potentially are involved in degradation of plant cell wall components, melanin formation and synthesis of secondary metabolites. Comparative genome analyses between members of different *R. solani* anastomosis groups, namely AG1-IA, AG3 and AG8 and the newly annotated *R. solani* AG1-IB genome were performed within the comparative genomics platform EDGAR. It appeared that only 21 to 28% of all genes encoded in the draft genomes of the different strains were identified as core genes. Based on Average Nucleotide Identity (ANI) and Average Amino-acid Identity (AAI) analyses, considerable sequence differences between isolates representing different anastomosis groups were identified. However, *R. solani* isolates form a distinct cluster in relation to other fungi of the phylum *Basidiomycota*. The isolate representing AG1-IB encodes significantly more genes featuring predictable functions in secondary metabolite production compared to other completely sequenced *R. solani* strains. The newly established *R. solani* AG1-IB 7/3/14 gene layout now provides a reliable basis for post-genomics studies.
Introduction

The basidiomycetous fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* [Frank] Donk) is a worldwide prevalent soil-borne plant pathogen. It causes diseases on many economically important crops including sugar beet, potato, rape, maize, soybean, rice, cabbage, cauliflower, tomato and lettuce as well as ornamental plants and forest trees [1]. Currently, *R. solani* isolates are classified into 13 distinct groups named anastomosis groups (AGs) based on occurrence of hyphal interaction between isolates of the same AG [2].

The genomic era for *R. solani* AG1 has been initiated by publication of the draft genome sequences for the AG1-IB isolate 7/3/14 [3,4] and the AG1-IA isolate B275 [5]. The genome sequences for AG3 and AG8 isolates became available quite recently [6,7]. The *R. solani* AG1-IB genome project provided first insights into the genome size and structure, gene content and functional annotation of the sequenced isolate. Moreover, the isolate’s diploid nature was followed at the genomic level. However, gene prediction for *R. solani* AG1-IB at that time was based on a gene model developed for the related fungus *Coprinopsis cinerea* of the order *Agaricales*, whereas *R. solani* belongs to the order *Cantharellales*. Recently, transcriptome sequence data obtained by high-throughput (HT) sequencing of Expressed Sequence Tags (ESTs) became available for *R. solani* AG1-IB [8]. *R. solani* ESTs provided information on genes transcribed under the conditions tested and their exon structure. Moreover, putative pathogenicity determinants were recognized within the EST dataset.

In general, gene prediction in fungal genomes is limited by the accuracy of gene prediction programs. *Ab initio* gene prediction for eukaryotes is based on methods that exploit structural and statistical properties of coding sequences that differ for each organism. Gene prediction rules are compiled from correctly recognized coding sequences of the organism of interest or one of its close relatives. Different gene prediction tools for eukaryotes have been developed over the last 30 years, e.g. GeneMark [9], GenomeScan [10], AUGUSTUS [11] and others. These tools are routinely applied for automatic gene finding in eukaryotic genomes. However, the performance of existing gene prediction tools still is unsatisfactory [11]. Recently, improvements in fungal gene prediction were achieved by using RNA-Seq and homology information implemented in the SnowyOwl pipeline [12]. Another important factor affecting gene model development for gene prediction is the degree of fragmentation in eukaryotic genome projects after assemblies. Frequently genes are split between contigs. However, reliable gene prediction algorithms and models are needed to enable comparative gene-based analyses between the genomes of related species and sub-species. Bioinformatics tools, such as the comparative genomics platform EDGAR [13], address the identification of orthologous genes in different genomes and the calculation of the core genome and unique genes for each genome within a set of genomes to be compared. Commonly, comparative genome analyses were undertaken to estimate unique genome features of an isolate of interest and to determine its relationship to related and reference strains. First comparative analyses of two *R. solani* isolates, namely those representing AG1-1A and AG1-IB, were recently published [14]. However, gene-based comparative analyses for all sequenced *R. solani* isolates representing different AGs have not been undertaken so far.

The objective of this study was to take advantage of the *R. solani* AG1-IB Expressed Sequence Tag dataset [8] as a valuable resource for improving recognition and prediction of genes in the genome of this isolate. In particular, an *R. solani* AG1-IB specific gene model was developed and applied to enable comparative genome analyses comprising other *R. solani* genome sequences, namely those of *R. solani* isolates classified as belonging to AG1-1A, AG1-IB, AG3 and AG8 [3-7]. Since the *R. solani* AGs included in this comparison differ in host specificity regarding their pathogenic interactions, it was hypothesized that unique genes...
within their genomes should reflect specific characteristics of the corresponding isolate. It was attempted to identify candidate genetic determinants that may play a role in host-specific pathogenicity, especially for *R. solani* AG1-IB. Moreover, application of the newly developed *R. solani* gene model was expected to enable recognition of new, so far missed genes in the *R. solani* AG1-IB genome and hence improvements regarding gene content and functional gene annotation were in the focus of this study.

**Material and Methods**

**Sequence datasets for the development of an *R. solani* AG1-IB specific gene model**

The improved genome sequence [EMBL: CDGK01000001–CDGK01018395 (Contigs); LN679100–LN679996 (Scaffolds)] [4] and the expressed sequence tag (EST) dataset [EMBL: HG330226-HG379789] [8] for *R. solani* AG1-IB (isolate 7/3/14) were used for the development of an AG1-IB specific gene model. Both datasets were established by applying the gsAssembler software (2.6/2.8).

**Mapping of ESTs onto the *R. solani* AG1-IB 7/3/14 genome for generation of a gene structure file**

The combined *R. solani* AG1-IB 7/3/14 EST dataset [8] was mapped onto the *R. solani* AG1-IB 7/3/14 genomic contigs as described previously using the mapping program GMAP [15] to identify gene encoding regions. For classification of alternative splicing events, the software tool ASTALAVISTA was used [16]. Finally, the manually curated output of GMAP, a gene structure file (.gff), was used as training set for the development of an *R. solani* AG1-IB 7/3/14 gene model.

**Gene model training, gene prediction based on the training dataset and evaluation of different gene models**

The gene structure file computed by means of GMAP was imported into the AUGUSTUS training system to deduce a parameter dataset for *R. solani* AG1-IB. A parameter dataset comprises Markov chain transition probabilities of coding (exon) and non-coding (intron or intergenic) regions. For each species, there are also 'meta parameters' such as the order of the Markov chain, or the size of the window used for the splice-site models. The parameter dataset includes species specific information such as intron and exon length distributions, splice-site patterns, translation start-site patterns or branch point regions of introns.

Gene prediction was accomplished by applying AUGUSTUS version 3.0.3 [11] on *R. solani* AG1-IB contigs by applying the newly developed gene model for this species. To evaluate gene prediction results, identified genes were compared to EST sequences by means of BLASTn [17], to unassembled transcriptome reads by means of bowtie2 [18] and to references gene products deposited in the NCBI database by means of BLASTp [17]. In addition, the gene prediction results were compared to the gene prediction based on the *C. cinerea* gene model. To verify intron-exon and exon-intron borders, the tool Geneious version 6.0.3 created by Biomatters (http://www.geneious.com/) was used for multiple alignments applying default settings. Transcript isotig mappings on *R solani* AG1-IB contigs were taken as references and alignment results were compared to the gene structure obtained by gene prediction. The *R. solani* AG1-IB gene prediction based on the specific parameter set is publicly available in the next version of AUGUSTUS and on the website http://bioinf.uni-greifswald.de/webaugustus/prediction/create. The newly predicted genes were annotated by means of the automatic annotation.
pipeline in SAMS [19,20] and a modified GenDB 2.0 version [20,21]. Mobile genetic elements belonging to the Long Terminal Repeat (LTR) group were identified and annotated by applying thirteen Hidden-Markov-Model (HMM) profiles of eight different protein domains (INT [PF14657, PF12835, PF00665, PF02920], RT [PF00078], AP [PF00847], RNase H [PF00075], Gag [PF00540, PF00607, PF08705, PF02093, PF02337, PF01141, PF01140, PF02228, PF03732, PF08723], Chromo [PF00385], RVT_thumb [PF06817], RVT_connect [PF06815]) by applying the tools LTRharvest [22] and LTRdigest [23].

Comparative genome analyses for R. solani isolates representing different anastomosis groups

Annotated genome information for the R. solani AG1-IA isolate B275 [GenBank: AFRT00000000], R. solani AG8 isolate WAC10335 [GenBank: AVOZ00000000] and R. solani AG3 isolate Rhs1AP [GenBank: JATN0100000] are publicly available. The genomes of these R. solani isolates were used for comparative genome analyses. Comparative analyses between the R. solani AG1-IB 7/3/14 draft genome and the R. solani genomes listed above were accomplished using a modified version of the comparative genomics program EDGAR designed to handle eukaryotic genomes and their multi-exon genes [13]. Comparative analyses comprised identification of orthologous genes and classification of genes as core genes or singletons.

Phylogenetic analysis of basidiomycetous fungi based on core genes

Phylogenetic relationships for R. solani AG1-IB 7/3/14, R. solani AG1-IA, R. solani AG3 and R. solani AG8 as well as the related fungi Coprinopsis cinerea okayama 7#130 [24], Piriformospora indica DSM 11827 [25] and Cryptococcus neoformans var. neoformans JEC21 [26] were computed by means of EDGAR [13]. The core genome of all selected fungi was calculated within EDGAR and based on all core genes, phylogenetic distances were calculated from multiple sequence alignments. Phylogenetic trees were constructed from concatenated core gene alignments using PHYLIP [27] as previously outlined in detail [13]. In addition, average nucleotide identity (ANI) and average amino acid identity analyses (AAI) were performed as described previously [28,29] to determine the relationship between R. solani isolates representing different anastomosis groups (AGs). For determination of thresholds regarding fungal species, ANI and AAI analyses were performed comprising the genomes of Aspergillus niger SH-2 (AUZU01), Aspergillus niger ATCC1015 (ACJE01), Candida albicans WO-1 (AAF001), Candida albicans A20 (AVAX01), Metarhizium anisopliae BRIP 53293 (APNC01), Metarhizium anisopliae BRIP 53284 (APNB01), Cryptococcus neoformans var. neoformans JEC21 (ACE17341-56), Cryptococcus neoformans var. neoformans B-3501A (CM000040-53), Fusarium oxysporum f.sp. cubense race 1 (AMGP01) and Fusarium oxysporum f.sp. cubense race 4 (AMGQ01).

Results and Discussion

Development of an R. solani AG1-IB gene model exploiting EST mapping results

The R. solani AG1-IB 7/3/14 draft genome sequence was established recently [3,4]. Likewise, Expressed Sequence Tags (ESTs) were deeply sequenced for this R. solani isolate grown in different media [8]. These sequence datasets (genomic and EST sequences) were now used to deduce an R. solani specific gene model which then was applied to uncover new R. solani AG1-IB genes that were missed in previous gene predictions on genomic contigs or are not
represented in the EST datasets. Accordingly, isotigs (transcript isoforms) from the *R. solani* AG1-IB 7/3/14 EST datasets were mapped onto the improved corresponding genome sequence to define constraints for the prediction of exon-intron and intron-exon junctions and to determine coding-region start-sites [8]. An *R. solani* gene model was computed by applying the eukaryotic gene prediction program AUGUSTUS [11] that previously was used in many fungal genome annotation projects, e.g. for the two *Basidiomycota* species *L. bicolor* [30] and *C. cinerea* [24]. Gene prediction in *R. solani* AG1-IB based on a specific gene model is the prerequisite for reasonable comparative analyses between the genomes of *R. solani* isolates representing different AGs.

Exact exon-intron junctions were deduced by mapping of isotig sequences from the EST datasets onto genomic sequences by means of the mapping tool GMAP. In total, 13,185 of 20,202 *R. solani* AG1-IB 7/3/14 isotigs were mapped onto the genome with more than 95% sequence identity and more than 90% template coverage. In most cases, only one isotig of a specific isogroup was mapped when strict mapping settings were applied. The following aspects may explain why 35% of the isotigs were not mapped on the *R. solani* genome: i) The genome assembly still is fragmented causing disruption of genes that are split between contigs. ii) Normalization in the course of library preparation for EST sequencing may have led to the enrichment of isotigs originating from rare nuclei that are not completely represented in the genome assembly. According to academic opinion, rare nuclei may be present due to the heterokaryotic nature of *R. solani* AG1-IB. However, the high degree of consistency between the genome assembly and the *de novo* transcriptome assembly revealed that the gsAssembler (version 2.6/2.8) is an appropriate tool for genome as well as transcriptome assemblies.

Visual inspection of aligned transcripts revealed alternatively spliced transcripts. Further analyses applying ASTALAVISTA [16], a bioinformatics tool for the analysis of alternative splicing events, uncovered that 4,796 alternative splicing events are represented by the mapped transcripts including 2,780 ‘intron-retention’ events, 239 ‘alternative acceptor-site’ events, 102 ‘alternative donor-site’ events, 12 ‘exon-skipping’ events and 1663 events that could not be classified into one of the aforementioned categories (Fig 1). These observations are in accordance with the findings of McGuire *et al.* [31] who showed that ‘intron-retention’ is the most prevalent alternative splicing event in fungi and that alternative acceptor-sites occur more frequently than alternative donor-sites. Transcriptome mapping results were retained in a file which then was applied as training file within AUGUSTUS for gene prediction on the *R. solani* AG1-IB 7/3/14 genome. This approach led to computation of an *R. solani* AG1-IB specific parameter set defining constraints such as translation initiation start sites, translation end points, acceptor (3’) splice sites, donor (5’) splice sites, exon and intron regions [32] which then were applied for gene prediction in this species.

The newly developed gene model improves gene prediction in the *R. solani* AG1-IB genome

Gene prediction results on *R. solani* AG1-IB genomic contigs applying the new *R. solani* AG1-IB specific parameter set were compared to those obtained with the *C. cinerea* parameter set as described previously [3]. In general, gene predictions based on the two different parameter sets featured different values in all categories (Table 1). For instance, 12,616 genes were predicted by applying the new model, whereas only 11,157 genes were recognized in the approach using the *C. cinerea* gene model.

To identify the most appropriate gene prediction approach, gene products deduced from predicted genes were analyzed with the program BLASTp in comparison to those deduced from EST isogroups by translation. In total, 9,595 of 10,101 gene products from the latter
approach were identified in the gene prediction based on the new gene model (94.99%), whereas only 86% of the gene products were identified using the *C. cinerea* gene model (Table 1). More than 3000 genes that are not represented within the EST dataset were predicted by application of the *R. solani* AG1-IB specific gene model. A total of 1256 of these so far unrecognized genes could be corroborated by mapping of non-assembled transcriptome reads

![Fig 1. Alternative transcript splicing in R. solani AG1-IB 7/3/14. Transcript isoforms for selected genes are shown. Each line with bars represents a transcript isoform. The pileup track below the isoforms shows the amount of reads that were mapped to the corresponding genomic region. (A) A gene for which only one transcript isoform was identified. Sharp intron-exon borders are visible. (B) Intron retention is the most common alternative splicing event observed in *R. solani* AG1-IB. (C) A gene whose transcript exists in three isoforms featuring exon-skipping as well as intron-retention.](https://example.com)

**Table 1. Comparison of gene prediction results applying the specific vs. the *C. cinerea* gene model on the *R. solani* AG1-IB 7/3/14 draft genome.**

| Features                                      | Specific gene model (based on *R. solani* AG1-IB gene structure file) | Previous gene model (C. cinerea model) |
|-----------------------------------------------|------------------------------------------------------------------------|----------------------------------------|
| Number of predicted genes                    | 12,616                                                                 | 11,157                                 |
| Average gene length                          | 1788 bp                                                                | 1541 bp                                |
| Average number of exons per gene             | 6.26                                                                   | 6.41                                   |
| Average exon length                          | 218.71 bp                                                              | 190.45 bp                              |
| Average intron length                        | 78.12 bp                                                               | 68.17 bp                               |
| CDSs matching an EST of the transcriptome dataset (%)\(^1\) | 9,595 (94.99%)                                                         | 8,702 (86.74%)                         |
| CDSs not matching an EST in the transcriptome dataset |                                                                         |                                        |
| CDSs having the same start and stop-position as predicted for the corresponding reference EST | 6,136                                                                  | 3,643                                  |

\(^1\) Gene model developed in this work based on GMAP mapping of *R. solani* AG1-IB Expressed Sequence Tags (ESTs)

\(^2\) Gene model applied in previous gene predictions for the *R. solani* AG1-IB genome [3]

\(^3\) 10,101 ESTs representing different isogroups (as described in chapter 3.1) correspond to 100%

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onto them. For the remaining 1765 new genes, 1289 homologous references genes were detected in the NCBI database. These mainly originate from *R. solani* AG3 and hence represent orthologous genes in both isolates. Only 476 new *R. solani* AG1-IB genes could neither be supported by EST-mapping nor by database homologs.

Based on Pfam annotation, most of the newly identified genes encode hypothetical proteins (~1400). However, also genes encoding cytochromes P450 and enzymes potentially involved in degradation of plant cell wall components were predicted (see S1 Table).

EST mappings also verify rules for exon-intron and intron-exon junctions as defined by the *R. solani* AG1-IB specific gene model. Gene predictions based on the two different gene models exemplarily are shown in Fig 2 for a genomic region represented by a selected contig. Application of the new gene model led to the identification of 5735 genes that were missed or insufficiently recognized in the previous approach. These genes were automatically annotated within the Sequence Analysis and Management System SAMS [19] as well as a modified GenDB 2.0 version [21]. These systems assigned annotations and features with high confidence values to 3,514 of 5,735 new *R. solani* AG1-IB 7/3/14 genes. However, most of the newly predicted genes received annotations such as ‘hypothetical protein’ or ‘uncharacterised protein’ illustrating insufficient functional characterization of fungal genomes from members of the genus *Rhizoctonia* in databases. SAMS and GenDB annotation pipelines assigned 185 gene names, 588 EC numbers and 1050 KOG numbers to the new *R. solani* AG1-IB 7/3/14 genes (S1 Table).

Most of the functionally annotated and newly identified genes belong to retrotransposons or retroviruses. Transposable elements with long terminal direct repeats (LTR TEs) are one of the extensively studied group of mobile genetic elements [33]. In total, 231 LTR retrotransposons were identified in the *R. solani* AG1-IB 7/3/14 genome by means of the LTRharvest tool [22]. Among these elements, 129 LTR retrotransposons represent the Ty3/Gypsy and 66 the Ty1/Copia type. Likewise, other members of the phylum *Basidiomycota* such as *Postia placentia*, *Laccaria bicolor*, *Coprinopsis cinerea* and *Phanerochaete chrysosporium* also mainly harbor Ty3/Gypsy LTR retrotransposons [33].

New genes encoding enzymes predicted to be involved in degradation of cellulosic material, lignocellulose and cutin, melanin formation and synthesis of other secondary metabolites were identified [8,34]. Moreover, genes for different cytochromes P450, non-ribosomal peptide-synthetases, a phenol reductase and enzymes involved in alkaloid synthesis potentially extend the functional context of toxin synthesis in *R. solani* AG1-IB.

To summarize, application of the *R. solani* AG1-IB specific gene model revealed 5735 so far non-recognized genes, some of which may have a function in the context of fungus-plant interaction.

**Comparative genome analyses for different *R. solani* isolates revealed remarkable differences between members representing anastomosis groups AG1, AG3 and AG8**

Four *R. solani* draft genome sequences representing AG1-IA [5], AG1-IB [3,4], AG3 [6] and AG8 [7] are available to date. A comparative analysis regarding genome features for these *R. solani* draft genomes is shown in Table 2. *R. solani* AG1-IA possesses the smallest draft genome that approximately is six million bases smaller than the *R. solani* AG1-IB 7/3/14 genome and only comprises two-thirds of the *R. solani* AG1-IB 7/3/14 gene content. The *R. solani* AG1-IB 7/3/14 mitochondrial (mt) genome also is 15 kb larger than the AG1-IA mt-genome and encodes seven additional genes. The genome of *R. solani* AG8 is slightly larger than that of isolate AG1-IA and has the smallest mt-genome of all sequenced *R. solani* strains. However, it has the highest coding density and the largest number of predicted genes within the set of
sequenced \textit{R. solani} isolates. \textit{R. solani} AG3 has the largest draft genome size, as well as the largest mitochondrial genome with a size of approximately 236 kb [35].

A gene-based comparative genome analysis for different \textit{R. solani} isolates belonging to the different AGs has not been undertaken before because of missing gene information for some isolates. Recently, specific gene predictions for all four \textit{R. solani} genomes became available. Gene-based comparisons comprising these annotated genome sequences were performed by means of the comparative genomics tool EDGAR [13] (Fig 3). It appeared that 2922 genes corresponding to 21 to 28\% of all genes identified in individual draft genomes represent the core set of genes present in all genomes analysed. Shared genes between sub-sets of \textit{R. solani} isolates are depicted in Fig 3. The genomes of the isolates AG1-IB and AG3 possess the largest set of shared genes. In contrast, AG1-IA and AG8 are more distantly related to each other. In first instance, all predicted core genes represent primary house-keeping genes that are expected to be encoded in all \textit{R. solani} genomes. In addition, also genes potentially involved in plant cell wall degradation were identified within the core-set of genes (S2 Table).

Comparative analyses also uncovered unique features of each \textit{R. solani} isolate (Table 3, S3, S4, S5 and S6 Tables). All isolates possess unique genes potentially involved in the production of plant cell wall degrading enzymes and synthesis of secondary metabolites, e.g. laccase,

### Table 2. Genome features of completely sequenced \textit{R. solani} isolates.

| Features                          | \textit{R. solani} AG1-IA\(^1\) | \textit{R. solani} AG1-IB\(^2\) | \textit{R. solani} AG3\(^3\) | \textit{R. solani} AG8\(^4\) |
|----------------------------------|---------------------------------|---------------------------------|----------------------------|-----------------------------|
| No. of scaffolds                 | 2,649                           | 879                             | 328                        | 857                         |
| Scaffold length                  | 37.09 Mb                        | 42.80 Mb                        | 51.71 Mb                   | 39.82 Mb                    |
| No. of CDSs                      | 10,489                          | 12,713                          | 12,726                     | 13,952                      |
| GC-content                       | 47.61\%                         | 48.10\%                         | 48.40\%                    | 48.80\%                     |
| Size of mitochondrial genome    | 147,264 bp                      | 162,751 bp                      | 235,849 bp                 | 139,993 bp                  |
| CDSs in mitochondrial genome    | 21                              | 28                              | 139                        | 53                          |
| GC content of mitochondrial genome | 33.93\%                      | 36.41\%                         | 35.91\%                    | 35.32\%                     |
| tRNAs within mitochondrial genome | 26                           | 25                              | 26                         | 17                          |

\(^1\) [5]  
\(^2\) [3,4]  
\(^3\) [6,35]  
\(^4\) [7]  

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In comparison to the other isolates, *R. solani* AG1-IB 7/3/14 harbours more genes with predictable functions in secondary metabolite synthesis, e.g. non-ribosomal peptide synthesis genes probably involved in siderophore production, terpene synthesis genes and tyrosinase genes having a putative function in melanin-production.

In summary, comparative genome analyses comprising the different completely sequenced *R. solani* isolates led to the identification of unique genome features for each isolate. Identified unique genes for different isolates are candidate determinants to explain differences in host range and virulence of corresponding *R. solani* isolates.

Table 3. Examples of predicted unique gene products for *R. solani* isolates representing AG1-IA, AG1-IB, AG3 and AG8.

|                        | *R. solani* AG1-IA | *R. solani* AG1-IB | *R. solani* AG3 | *R. solani* AG8 |
|------------------------|--------------------|--------------------|----------------|----------------|
| Cellulolytic enzymes<sup>1</sup> | 34                 | 76                 | 95             | 76             |
| Tyrosinases<sup>1</sup>       | 2                  | 10                 | 12             | 9              |
| Drug resistance proteins<sup>1</sup> | 19                | 10                 | 7              | 5              |
| Laccases<sup>1</sup>          | 4                  | 8                  | 22             | 13             |
| Cytochrome P450<sup>1</sup>   | 46                 | 95                 | 48             | 38             |
| Non-ribosomal peptide synthesis enzymes<sup>1</sup> | 0                  | 2                  | 0              | 0              |
| Alkaloid/terpene synthesis enzymes<sup>1</sup> | 0                  | 2                  | 3              | 2              |
| Tannases<sup>1</sup>          | 1                  | 5                  | 4              | 1              |
| Volvatoxin-like<sup>1</sup>   | 0                  | 0                  | 1              | 0              |

<sup>1</sup> Corresponding genes were listed in [S7 Table](#)

**Fig 3.** Venn diagram of the gene-based genome comparison for the completely sequenced *R. solani* isolates representing AG1-IA, AG1-IB, AG3 and AG8. The core genome of all isolates consists of 2922 genes. These genes are present in the genomes of all sequenced *R. solani* isolates: *R. solani* AG1-IB 7/3/14 [EMBL:CDGK01000001-CDGK01018395 (Contigs); LN679100-LN679999 (Scaffolds)], *R. solani* AG3 [GenBank: JATN0100000], *R. solani* AG1-IA [Genbank: AFRT00000000] and *R. solani* AG8 [GenBank: AVOZ0000000]. For computation of the Venn diagram, default settings of EDGAR [13] were applied.
Phylogenetic classification of *R. solani* isolates based on shared core genes

To deduce the phylogeny of the different *R. solani* isolates in relation to other completely sequenced members of the phylum *Basidiomycota*, the comparative genomics tool EDGAR was applied. Based on 725 core genes determined for selected species, a phylogenetic tree was computed (Fig 4). The topology of the resulting tree is congruent to the tree calculated for the 18S rRNA marker gene [3]. *R. solani* isolates representing the anastomosis groups AG1-IA, AG1-IB, AG3 and AG8 cluster together. *R. solani* AG1-IB and *R. solani* AG1-IA are more closely related to each other than to *R. solani* AG3 and *R. solani* AG8. The other fungi of the phylum *Basidiomycota* included in the phylogenetic analysis are only distantly related to *R. solani*. *C. cinerea* and *P. indica* cluster within one group, whereas *C. neoformans* only is distantly related to the other fungi. These results are in agreement with previous taxonomic classifications. *C. cinerea* and *P. indica* belong to the same class (*Agaricomycetes*), whereas *C. neoformans* is a member of the class *Tremellomycetes*. To determine similarities within the *R. solani* species complex, pairwise Average Nucleotide Identities (ANI) and Average Amino-acid Identities (AAI) were calculated (Table 4 & 5). Usually, genomes of prokaryotic isolates

**Table 4. Pairwise Average Nucleotide Identity (ANI) analyses for completely sequenced *R. solani* isolates.**

|         | AG1-IA   | AG1-IB   | AG3     | AG8     |
|---------|----------|----------|---------|---------|
| AG1-IA  | 100.00%  | 81.82%   | 79.23%  | 79.09%  |
| AG1-IB  | -        | 100.00%  | 79.29%  | 79.22%  |
| AG3     | -        | -        | 100.00% | 84.07%  |
| AG8     | -        | -        | -       | 100.00% |

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belonging to the same species possess higher ANI and AAI values (above 95%) than those representing different species [28,29].

Pairwise comparisons for *R. solani* isolates revealed ANI and AAI values below 88% for each pair, indicating considerable sequence differences between isolates representing different anastomosis groups. As a control, ANI and AAI values were also calculated for other fungal strains belonging to the same species (s. Table 6). In these cases, ANI and AAI values above 97% were obtained for isolates representing the same species. However, it should be noted that corresponding genomes were sequenced in their haploid state accounting for a more homogeneous genome shape compared to the diploid (heterozygous) state of the *R. solani* genomes involving a higher degree of heterogeneity.

However, ANI/AAI values below 88% for pairwise *R. solani* comparisons tentatively may suggest that *R. solani* isolates representing different anastomosis groups diverged substantially and probably form distinguishable lineages.

**Concluding remarks**

Several draft genome sequences for different fungi were established recently by taking advantage of high-throughput sequencing technologies. Application of bioinformatics tools for sequence analysis and interpretation of fungal genomes provided insights into their gene content and life style. However, gene prediction and comparative analyses are still insufficient for this group of microorganisms which mainly is due to the lack of specific gene models for different species and functional gene analyses. Expressed Sequence Tag (EST) and/or high-throughput transcriptome sequencing has only been accomplished for a limited number of species. Hence, application of non-homologous gene models led to inaccuracies in gene predictions. Therefore, important information within fungal genome projects still remains unexplored.

In this work, a new, manually evaluated gene model for an *R. solani* AG1-IB isolate was developed and enabled improvements regarding gene prediction and comparative genome analyses among members of the *R. solani* species complex. The total number of predicted *R. solani* AG1-IB genes may not reflect the complete set of genes for this isolate which mainly is due to the high degree of fragmentation within the obtained draft genome sequence. Genes that are split between contigs cannot be recognized correctly. The multi-cellular and diploid
nature of R. solani AG1-IB 7/3/14 most probably complicates concise genome assemblies and hence detection of the full set of genes within its genome is demanding. Similar problems were reported for other eukaryotic genome projects [36]. Sequencing of the haploid genome status cannot be addressed for R. solani, since the corresponding phase in its life cycle has not been recognized so far. However, the improved and complemented gene set achieved by application of the R. solani AG1-IB specific gene model is expected to comprise the great majority of genes encoded in this isolate.

Subsequently to the new gene prediction and annotation of the R. solani AG1-IB genome, a comparative genome analysis for R. solani isolates representing different anastomosis groups was enabled. Significant differences between R. solani genomes were uncovered and now provide the genomic basis for studies addressing host-specificity in pathogenic interactions.

The newly predicted R. solani AG1-IB 7/3/14 gene set is a valuable resource for evaluation of further RNA-Seq experiments to analyze the pathogenic interaction of the fungus with its host plant. First insights into possible pathogenicity determinants were obtained by R. solani AG1-IB 7/3/14 high-throughput transcriptome sequencing [8]. However, RNA-Seq analyses for R. solani AG1-IB in interaction with its host plant lettuce will provide deeper insights into differential transcription of candidate pathogenicity genes. Finally, a better understanding of the function of R. solani pathogenicity factors is a prerequisite for the development of strategies to control plant diseases caused by this fungus.

Supporting Information

S1 Table. Newly predicted genes in the R. solani AG1-IB 7/3/14 genome by application of the R. solani specific gene model. The table provides information on newly predicted genes regarding Gene name, Gene product, Gene function, EC Number, KOG number and KOG functional categories as annotated within the annotation platform GenDBE. (XLS)

S2 Table. R. solani core genes. The table provides information on KOG and PFAM assignments for all R. solani core genes. (XLS)

S3 Table. Singleton genes of R. solani AG1-IA. The table provides information on KOG and PFAM assignments for all R. solani AG1-IA singleton genes. (XLS)

S4 Table. Singleton genes of R. solani AG1-IB. The table provides information on KOG and PFAM assignments for all R. solani AG1-IB singleton genes. (XLS)

S5 Table. Singleton genes of R. solani AG3. The table provides information on KOG and PFAM assignments for all R. solani AG3 singleton genes. (XLS)

S6 Table. Singleton genes of R. solani AG8. The table provides information on KOG and PFAM assignments for all R. solani AG8 singleton genes. (XLS)

S7 Table. Genes of interest identified in the R. solani AG1-IA, AG3 and AG8 isolates. The table provides information on KOG and PFAM assignments for genes of interest identified in R. solani AG1-IA, AG3 and AG8 isolates. (XLS)
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Author Contributions

Conceived and designed the experiments: RG AP AS. Performed the experiments: DW MK BV RG. Analyzed the data: DW OR JB LJ RG AS. Contributed reagents/materials/analysis tools: JB AG SA. Wrote the paper: DW RG AP AS. Software development: JB LJ.

References

1. González García V, Portal Onco MA, Rubio Susan V. Review. Biology and Systematics of the form genus Rhizoctonia. Spanish J Agric Res. 2006; 4: 55–79.
2. Carling DE, Baird RE, Gitaitis RD, Brainard KA, Kuninaga S. Characterization of AG-13, a Newly Reported Anastomosis Group of Rhizoctonia solani. Phytopathology. 2002; 92: 893–899. doi:10.1094 PHYTO.2002.92.8.893 PMID: 18942969
3. Wibberg D, Jelonek L, Rupp O, Hennig M, Eikmeyer F, Goesmann A, et al. Establishment and interpretation of the genome sequence of the phytopathogenic fungus Rhizoctonia solani AG1-IB isolate 7/3/14. J Biotechnol. 2013; 167: 142–155. doi: 10.1016/j.jbiotec.2012.12.010 PMID: 23280342
4. Wibberg D, Rupp O, Jelonek L, Kröber M, Verwaajen B, Blom J, et al. Improved genome sequence of the phytopathogenic fungus Rhizoctonia solani AG1-IB 7/3/14 as established by deep mate-pair sequencing on the MiSeq (Illumina) system. J Biotechnol. 2015; 203: 19–21. doi: 10.1016/j.jbiotec.2015.03.005 PMID: 25801332
5. Zheng A, Lin R, Zhang D, Qin P, Xu L, Ai P, et al. The evolution and pathogenic mechanisms of the rice sheath blight pathogen. Nat Commun. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013; 4: 1424.
6. Cubeta MA, Thomas E, Dean RA, Jabaji S, Neate SM, Tavantzis S, et al. Draft Genome Sequence of the Plant-Pathogenic Soil Fungus Rhizoctonia solani Anastomosis Group 3 Strain Rhs1AP. Genome Announc. 2014; 2.
7. Hane JK, Anderson JP, Williams AH, Sperschneider J, Singh KB. Genome Sequencing and Comparative Genomics of the Broad Host-Range Pathogen Rhizoctonia solani AG8. PLoS Genet. 2014; 10.
8. Wibberg D, Jelonek L, Rupp O, Kröber M, Goesmann A, Grosch R, et al. Transcriptome analysis of the phytopathogenic fungus Rhizoctonia solani AG1-IB 7/3/14 applying high-throughput sequencing of expressed sequence tags (ESTs). Fungal Biology. Jul 2014: 800–813. doi: 10.1016/j.funbio.2014.06.007 PMID: 25209639
9. Besemer J, Borodovsky M. GeneMark: web software for gene finding in prokaryotes, eukaryotes and viruses. Nucleic Acids Res. Oxford University Press; 2005; 33: W451–W454.
10. Yeh R-F, Lim LP, Burge CB. Computational Inference of Homologous Gene Structures in the Human Genome. Genome Res. Cold Spring Harbor Laboratory Press; 2001; 11: 803–816.
11. Stanke M, Keller O, Gunduz I, Hayes A, Waack S, Morgenstern B. AUGUSTUS: ab initio prediction of alternative transcripts. Nucleic Acids Res. Oxford University Press; 2006; 34: W435–W439.
12. Reid I, O’Toole N, Zabaneh O, Nourzadeh R, Dahdouli M, Abdellateef M, et al. SnowyOwl: accurate prediction of fungal genes by using RNA-Seq and homology information to select among ab initio models. BMC Bioinformatics. 2014; 15: 229. doi: 10.1186/1471-2105-15-229 PMID: 24980894
13. Blom J, Albaum SP, Doppmeyer D, Pühler A, Vorhölter F-J, Zakrzewski M, et al. EDGAR: A software framework for the comparative analysis of prokaryotic genomes. BMC Bioinformatics. BioMed Central; 2009; 10: 154.
14. Ghosh S, Gupta SK, Jha G. Identification and functional analysis of AG1-IA specific genes of Rhizoctonia solani. Curr Genet. 2014; 60: 327–41. doi: 10.1007/s00294-014-0438-x PMID: 25070039
15. Wu TD, Watanabe CK. GMAP: a genomic mapping and alignment program for mRNA and EST sequences. Bioinformatics. Oxford University Press; 2005; 21: 1859–1875.
16. Foissac S, Sammeth M. ASTALAVISTA: dynamic and flexible analysis of alternative splicing events in custom gene datasets. Nucleic Acids Res. Oxford University Press; 2007; 35: W297–W299.
17. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. Oxford Univ Press; 1997; 25: 3389–3402.

18. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature Methods. 2012. pp. 357–359. doi: 10.1038/nmeth.1923 PMID: 22388286

19. Bekel T, Henckel K, Küster H, Meyer F, Mittard Runte V, Neuweger H, et al. The Sequence Analysis and Management System—SAMS-2.0: Data management and sequence analysis adapted to changing requirements from traditional sanger sequencing to ultrafast sequencing technologies. J Biotechnol. 2009; 140: 3–12. PMID: 19297685

20. Rupp O, Becker J, Brinkrolf K, Timmermann C, Borth N, Pühler A, et al. Construction of a public CHO cell line transcript database using versatile bioinformatics analysis pipelines. PLoS One. 2014; 9.

21. Meyer F, Goesmann A, McHardy AC, Bartels D, Bekel T, Clausen J, et al. GenDB—an open source genome annotation system for prokaryote genomes. Nucleic Acids Res. 2003; 31: 2187–2195. PMID: 12682369

22. Ellinghaus D, Kurtz S, Willhoefif U. LTRharvest, an efficient and flexible software for de novo detection of LTR retrotransposons. BMC Bioinformatics. 2008; 9: 18. doi: 10.1186/1471-2105-9-18 PMID: 18194517

23. Steinbiss S, Willhoefif U, Gremme G, Kurtz S. Fine-grained annotation and classification of de novo predicted LTR retrotransposons. Nucleic Acids Res. 2009; 37: 7002–7013. doi: 10.1093/nar/gkp759 PMID: 19786494

24. Stajich JE, Wilke SK, Ahrén D, Au CH, Birrer BW, Borodovsky M, et al. Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom Coprinopsis cinerea (Coprinus cinereus). Proc Natl Acad Sci. National Acad Sciences; 2010; 107: 11889–11894.

25. Zuccaro A, Lahrmann U, Güldener U, Langen G, Pfiffi S, Biedenkopf D, et al. Endophytic Life Strategies Decoded by Genome and Transcriptome Analyses of the Mutualistic Root Symbiont Piriformospora indica. PLoS Pathog. Public Library of Science; 2011; 7.

26. Loftus BJ, Fung E, Roncaglia P, Rowley D, Amedeo P, Bruno D, et al. The genome of the basidiomycetous yeast and human pathogen Cryptococcus neoformans. Science (80-). 2005; 307: 1321–1324.

27. Felsenstein J. PHYLIP (Phylogeny Inference Package) [Internet]. University of Washington Seattle Department of Genome Sciences. Department of Genome Sciences, University of Washington; 2005.

28. Konstantinidis KT, Tiedje JM. Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci U S A; 2005; 102: 2567–2572. PMID: 15701695

29. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol. 2007; 57: 81–91. PMID: 17220447

30. Martin F, Aerts A, Ahren D, Brun A, Danchin EGJ, Duchaussoy F, et al. The genome of the basidiomycetous yeast and human pathogen Cryptococcus neoformans. Science (80-). 2005; 307: 1321–1324.

31. McGuire AM, Pearson MD, Neafsey DE, Galagan JE. Cross-kingdom patterns of alternative splicing and splice recognition. Genome Biol. 2008; 9: R50. doi: 10.1186/gb-2008-9-3-r50 PMID: 18321378

32. Stanke M, Morgenstern B. AUGUSTUS: A web server for gene prediction in eukaryotes that allows user-defined constraints. Nucleic Acids Res. 2005; 33.

33. Schäfer W. The role of cutinase in fungal pathogenicity. Trends Microbiol. 1993; 1: 69–71. PMID: 8044466

34. Losada L, Pakala SB, Fedorova ND, Joardar V, Shabalina SA, Hostetler J, et al. Mobile elements and mitochondrial genome expansion in the soil fungus and potato pathogen Rhizoctonia solani AG-3. FEMS Microbiology Letters. 2014. pp. 165–173. doi: 10.1111/1574-6968.12387 PMID: 24461055

35. Satou Y, Mineta K, Ogasawara M, Sasakura Y, Shoguchi E, Ueno K, et al. Improved genome assembly and evidence-based global gene model set for the chordate Ciona intestinalis: new insight into intron and operon populations. Genome Biol. BioMed Central; 2008; 9: R152.