Genomes and virulence difference between two physiological races of *Phytophthora nicotianae*

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**Abstract**

**Background:** Black shank is a severe plant disease caused by the soil-borne pathogen *Phytophthora nicotianae*. Two physiological races of *P. nicotianae*, races 0 and 1, are predominantly observed in cultivated tobacco fields around the world. Race 0 has been reported to be more aggressive, having a shorter incubation period, and causing worse root rot symptoms, while race 1 causes more severe necrosis. The molecular mechanisms underlying the difference in virulence between race 0 and 1 remain elusive.

**Findings:** We assembled and annotated the genomes of *P. nicotianae* races 0 and 1, which were obtained by a combination of PacBio single-molecular real-time sequencing and second-generation sequencing (both HiSeq and MiSeq platforms). Gene family analysis revealed a highly expanded ATP-binding cassette transporter gene family in *P. nicotianae*. Specifically, more RxLR effector genes were found in the genome of race 0 than in that of race 1. In addition, RxLR effector genes were found to be mainly distributed in gene-sparse, repeat-rich regions of the *P. nicotianae* genome.

**Conclusions:** These results provide not only high quality reference genomes of *P. nicotianae*, but also insights into the infection mechanisms of *P. nicotianae* and its co-evolution with the host plant. They also reveal insights into the difference in virulence between the two physiological races.

**Keywords:** Black shank, *Phytophthora nicotianae*, Genomes, Hybrid assembly, RxLR effector
the two races is affected by additional genetic factors [7].
To discover better and more efficient ways to control the
pathogen, we undertook a global examination of the genes
involved in the infection process from different races.
Although five strains of *P. parasitica* are already public
available [8], none of these includes any of the four phy-
siological races of *P. nicotianae*. Here we report the
genomes of *P. nicotianae* physiological races 0 and 1,
sequenced using a combination of PacBio single-molecule
real-time (SMRT) sequencing technology, and Illumina
HiSeq and MiSeq sequencing technologies, and identify
candidate genes that may cause the difference in virulence
between them.

**Isolation of *P. nicotianae* races and genomic DNA
extraction**

Tobacco plants infected by either *P. nicotianae* race 0 or
race 1 were obtained from Yunnan Tobacco Research
Institute. Any surface dirt on the infected plant was
washed off under tap water. After drying, stem tissue
from the lesion margin were cut into 5 × 5 mm squares,
sterilized using 70 % ethanol for 1 minute, and then
rinsed three times using sterile water. Sterilized tissue
squares were then placed in lima bean agar (LBA) plates
amended with 50 μg/ml ampicillin, 100 μg/ml rifampi-
cin, and 50 μg/ml of pentachloronitrobenzene to sup-
press possible contaminant. LBA plates were incubated
for 2–3 days in darkness at 25 °C. Color and texture of
the colony and mycelium were used to confirm the iden-
tity of *P. nicotianae*. Mycelium was transferred to LBA
slants and cultured for 7 days in darkness at 25 °C. Gen-
omic DNA was extracted using the modified cetyltrim-
methyl ammonium bromide method [9].

**Sequencing and quality control**

Whole-genome sequencing yielded 41 Gb HiSeq paired-
end reads, 5 Gb HiSeq mate pair reads, 5 Gb MiSeq
reads, and 5 Gb PacBio long reads for race 0; and 46 Gb
HiSeq pair-end reads, 4 Gb HiSeq mate pair reads, 3 Gb
MiSeq reads, and 6 Gb PacBio long reads for race 1
(Table 1). Illumina HiSeq reads were first filtered out
with >10 % N or with >40 bp low quality bases. Redund-
ant reads resulting in duplicate base calls were filtered
at a threshold of a Euclid distance ≤ 3 and a mismatch
rate of ≤ 0.1. Where duplicated paired-end (PE) reads
were identical, only one copy was retained. For adapter
contamination caused by DNA adapter dimerization,
empty loading, or too small an insert size (less than a
read length), we filtered out if both read 1 and read 2
contained an adapter ≥ 10 bp with a mismatch rate ≤ 0.1.
For PacBio reads, we first used the HGAP (SMRT Ana-
lysis v2.1.1) pipeline to perform self-correction (default
parameters). Longer PacBio reads were selected automo-
tically as seeds; the rest of the reads were aligned
against these seed sequences for correction. For hybrid
correction, we used LSC [10] (v1.0 alpha) with the par-
parameter for bowtie2 set to very-fast; pacBioToCA [11]
(wgs v8.0) using the parameter length 500. Corrected
PacBio long reads were obtained by aligning high accur-
cy HiSeq short reads against PacBio long reads. We
also used ECTools (July 6th 2014) to correct PacBio long
reads from both *P. nicotianae* races 0 and 1. ECTools
aligned unitigs assembled from MiSeq reads against
PacBio long reads to perform correction.

**Assembly**

Because of its relatively high heterozygosity, we used a
hybrid assembly approach to assemble the genome of
race 0 (Fig. 1). We also compared the performance
of different assemblers including Velvet (v1.2.09) [12],
ABySS (v3.81) [13], JR-Assembler (v1.0.3) [14], EULER-SR
(v1.1.2) [15], SPAdes (v3.0.0) [16], SOAPdenovo2 (r240)
[17], Celera Assembler (v8.0) [18] and Minimus2 (v 3.1)
[19] on *P. nicotianae* race 0. Comparison showed that
assemblies from PacBio reads were generally of better
quality than those from HiSeq reads (Fig. 2). The final as-
sembled genome sizes for race 0 and race 1 were 80 Mb
and 69 Mb, respectively, which is slightly different from

**Table 1** Sequencing and data size of *P. nicotianae* races 0 and race 1

| Races            | Library type  | Instrument | Fragment size (bp) | Read length (bp) | Data (Gb) Before quality control | Data (Gb) After quality control |
|------------------|---------------|------------|--------------------|------------------|----------------------------------|---------------------------------|
| *P. nicotianae* race 0 | Illumina paired-end | HiSeq      | 350                | 100              | 41                               | 34                              |
|                   | Illumina mate pair | HiSeq      | 2,000              | 100              | 5                                | 3                               |
|                   | Illumina paired-end | Miseq     | 500                | ~300             | 5                                | a3                              |
|                   | SMRTbell         | PacBio RS  | 10,000             | ~1,932           | 5                                | b3                              |
| *P. nicotianae* race 1 | Illumina paired-end | HiSeq      | 350                | 100              | 46                               | 20                              |
|                   | Illumina mate pair | HiSeq      | 2,000              | 100              | 4                                | 2                               |
|                   | Illumina paired-end | Miseq     | 500                | ~300             | 3                                | a2                              |
|                   | SMRTbell         | PacBio RS  | 10,000             | ~2,333           | 6                                | b4                              |

aTotal base pairs of Miseq reads after merged using Flash
bPacBio reads after correction with LSC.
Fig. 1 Assembly pipeline for *P. nicotianae* race 0. CA indicates Celera Assembler. Because of high heterozygosity of spores in *P. nicotianae* race 0, we used a hybrid approach including Celera Assembler, ABySS assembler, and Minimus2 to assemble this genome.

Fig. 2 NGx plot for different assemblies. Contigs of length equal to or above NGx occupy x% of the reference genome. pacBioToCA + LSC + HGAP refers to assembly from non-redundant corrected PacBio reads of LSC, pacBioToCA and HGAP, pacBioToCA + ABySS means assembly using pacBioToCA corrected reads and unitigs assembled by ABySS. Assembly from pacBioToCA + ABySS was also merged with assembly from ECTools corrected PacBio reads to generate the final assembly, which was then used for annotation. As illustrated, the NG50 for hybrid assemblies such as pacBioToCA + ABySS were longer.
the previous estimation of *P. nicotianae* (90 Mb) [20]. The corresponding contig N50 sizes were 23 kbp and 30 kbp, respectively (Table 2). Over 95% of core eukaryotic genes could be mapped to the two genomes using CEGMA [21], and over 90% of Illumina HiSeq reads could be mapped back to the genome assemblies.

**Annotation**

Known transposable elements (TEs) were identified with RepeatMasker (version 3.2.6) [22] using the Repbase TE library (v16.10) [23] and default parameters. Tandem repeats were predicted using TRF [24]. *gypsy* and *copia* types of long terminal repeat (LTR) were the main contributors to the repeat, making up 12.5% and 3.5% of the genome for race 0, and 11.5% and 3.6% of the genome for race 1. For gene structure prediction, gene sets from 9 species including *Phytophthora infestans* [25], *Phytophthora sojae* [26], *Phytophthora ramorum* [26], *Hyaloperonospora arabidopsi* [27], *Pythium aphanidermatum* [28], *Pythium arrhenomanes* [28], *Pythium irregulare* [28], *Pythium vexans* [28], *Pythium iwayamae* [28] and *Pythium ultimum* [29] were used for homology-based prediction. GENSCAN [30], AUGUSTUS [31] and GlimmerHMM [32] were used for de novo gene prediction. Evidence derived from homology-based and de novo predictions were then integrated in GLEAN to generate a consensus gene set. A total of 17,797 and 14,542 protein-coding genes were annotated in *P. nicotianae* race 0 and race 1, respectively. Over 97% of these genes could be aligned against KEGG [33], Swiss-Prot and TrEMBL databases [34]. Mean exon numbers per gene in *P. nicotianae* and related species varied between 2.2 and 2.8, suggesting that homology and de novo-based prediction were appropriate for annotation (Additional file 1). We also used publicly available expressed sequence tags (ESTs) from the appressorium [35] and mycelium [36, 37] of *P. nicotianae*.
to validate the annotation. We retrieved a total of 10,524 ESTs from the dbEST database. Using the threshold of match length >200 bp and E-value <1e-5, we aligned 8,043 ESTs to the race 0 genome and 7,618 ESTs to the race 1 genome. Additionally, 4,454 genes in race 0 and 3,604 genes in race 1 were supported by at least one EST (Additional file 2). Whole genome comparison using NUCmer [38] found that average identity was 99 % for 1-to-1 alignment, and 98.84 % for m-to-m alignment between P. nicotianae races 0 and 1. Using KaKs_Calculator, mean synonymous mutation ratio (Ks) was estimated to be 0.075 between race 0 and race 1 [39], and four genes were identified to be positively selected (Additional file 3).

Gene family clustering and evolution

Gene family clustering using OrthoMCL [40] revealed that over 72 % of gene families were shared between species pairs among P. nicotianae race 0, race 1, and related species (Additional file 4). The average number of genes per gene family was 1.19 to 1.50 in Phytophthora and 1.14 to 1.26 in Pythium, suggesting more copies of genes exist in the Phytophthora genus. A total of 1,604 single-copy genes were identified between P. nicotianae and the other 9 related species (Additional file 5). Gene family expansion and contraction estimated using CAFÉ [41] found that 1,237 gene families expanded and 294 gene families contracted in race 0, while 217 gene families expanded and 508 gene families contracted in race 1 (Fig. 3).

ABC transporter expanded in P. nicotianae

The ATP-binding cassette transporter (ABC transporter) superfamily facilitates the transport of ions, proteins, lipids and toxins across plant membranes [42]. Interestingly, a domain-centric study found this gene family to be enriched in the oomycete plant pathogen genomes [43]. It was proposed that an important function of ABC transporters in pathogens involves exporting toxic phytoalexins [44, 45]. Based on the result of CAFÉ analysis, we found the ABC transporter gene family to be significantly expanded in the branch of P. nicotianae (likelihood ratio test, p-value < 0.05), but not in the branch of

Fig. 4 Bin plot showing repeat and gene density distribution. Bins of gene density were sorted and plotted on the basis of 5’ and 3’ intergenic border lengths. The color of each bin represents the number of genes. a Distribution in P. nicotianae race 0 RxLR effectors. b Distribution of P. nicotianae race 0 genes. c Distribution of P. nicotianae race 1 RxLR effectors. d Distribution of P. nicotianae race 1 genes
**P. infestans** (likelihood ratio test, p = 0.9). To verify this result, we used Pfam to annotate ABC transporter domains (PF00005.22, PF00664.18, PF01061.19) between **P. infestans**, and **P. nicotianae** races 0 and 1 (Additional file 6). The portions of ABC transporters in **P. nicotianae** were significantly larger than that in **P. infestans** (chi-square test, p < 0.05). This result suggests that the ABC transporter family plays important roles in **P. nicotianae** in its adaptive evolution to the host.

**Distribution of effectors and their differences in races 0 and 1**

Plant pathogens have evolved to secrete effectors, which can manipulate the host immune system and suppress host defense. Based on their target sites in the host plant, effectors can be classified into two classes: (1) apoplastic effectors, which are secreted into plant extracellular spaces; and (2) cytoplasmic effectors, which are translocated into the plant cell. Some effector genes, e.g. **ATRS** in *H. arabidopisidis*, are found to be avirulence genes [46]. These genes are under selective pressure to evade host recognition while maintaining their original functions.

RxLR effectors are important cytoplasmic effectors that contain a conserved N-terminal motif (Arg-X-Leu-Arg). The RxLR motif is involved in translocation into host cells [47]. During infection, the RxLR family functions to suppress host immunity. This process usually involves manipulating plant immunity-associated signaling pathways. For example, PexRD2 can perturb MAPKKK signaling pathways to suppress NB-LRR-mediated immunity in **P. infestans** [48]. In addition, a set of RxLR effectors from **P. infestans** can suppress the signaling pathway induced by flg22, a kind of microbe-associated molecular pattern (MAMP) [49], while some RxLR effectors such as **Avr1b-1** and **Avr1k** can be recognized by NB-LRR immune receptors to confer resistance [50]. Research in 2015 also found that the evolution of RxLR effectors varies between the genus *Phytophthora* and downy mildews: more conserved RxLR effectors were observed in the genus *Phytophthora* [51]. We performed a whole-genome scan for RxLR effector genes in race 0 and race 1. The analysis showed that most RxLR genes were distributed in repeat-rich, gene-sparse regions (Fig. 4), suggesting rapid evolution of RxLR effectors. Specifically, a total of 308 RxLR effector genes were predicted in race 0, and 199 genes in race 1 (Additional files 7 and 8). The difference in RxLR effector gene numbers between race 0 and race 1 may contribute to the variance in the aggressiveness of these pathogens in cultivated tobacco.

Crinkler (CRN) effectors are another important class of effectors that cause leaf crinkling in plants [52]. To investigate CRN effectors in **P. nicotianae** races 0 and 1, we first used EMBOSS getorf (~minsize 300) to extract open reading frames (ORFs) from the whole genome, and then used HMMER (~E 1e-5) with existing profiles [25]. Predicted CRN effectors were filtered by the presence of the LxLFLAK motif. A total of 32 and 26 CRN effectors were annotated in **P. nicotianae** races 0 and 1, respectively. However, the number of CRN effectors may be underestimated, given the model we used [53].

**Availability of supporting data**

The genome assembly, annotation and sequencing reads of each sequencing library are available in the NCBI repository, project ID PRJNA294216. The genome assembly and annotation can also be accessed via the *GigaScience* GigaDB database [54].

**Additional files**

- **Additional file 1:** Statistics of gene lengths of **P. nicotianae** races 0 and 1, and related species. Statistics of gene lengths of **P. nicotianae** races 0 and 1, and related species. (XLS 503 kb)
- **Additional file 2:** Blast hits of ESTs on **P. nicotianae** races 0 and 1. Blast results of ESTs on **P. nicotianae** races 0 and 1. (TXT 2187 kb)
- **Additional file 3:** Positive selected genes between **P. nicotianae** races 0 and 1. Positive selected genes between **P. nicotianae** races 0 and 1, identified using KaKs_Calculator. (XLS 518 kb)
- **Additional file 4:** Venn diagram of gene family clustering between **P. infestans**, **P. ramorum**, and **P. nicotianae** races 0 and 1. Gene family clustering result from **P. infestans**, **P. ramorum**, and **P. nicotianae** races 0 and 1. A total of 7,552 conserved gene families among the four genomes were identified. (PDF 146 kb)
- **Additional file 5:** Gene families in **P. nicotianae** races 0 and 1, and related species. Total and single copy gene families in **P. nicotianae** races 0 and 1, and related species. (XLS 6019 kb)
- **Additional file 6:** Pfam annotation result of ABC transporter genes. Pfam annotation result of ABC transporter genes in **P. infestans**, and **P. nicotianae** races 0 and 1. (TXT 2775 kb)
- **Additional file 7:** List and amino acid sequences of RxLR and Crinkler effectors. List and amino acid sequences of RxLR and Crinkler effectors. (XLS 228 kb)
- **Additional file 8:** Comparison of number of effectors between **P. nicotianae**, and related species. Comparison of number of RxLR and Crinkler effectors between **P. nicotianae**, and related species. (XLS 59 kb)

**Abbreviations**

ABC transporter: ATP-binding cassette transporter; CRN: Crinkler effector; EST: expressed sequence tag; LBA: lima bean agar; LTR: long terminal repeat; ORF: open reading frame; PE: paired-end; RxLR: effectors with Arg-X-Leu-Arg motif; SMRT: single-molecular real-time sequencing.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

YD, XM, BX, and HL designed the study. HY isolated the races. XW extracted DNA. HL assembled the genomes and performed analyses. HL, HY, YD, BX wrote the manuscript. All authors read and approved the final manuscript.

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