Pan-Parastagonospora Comparative Genome Analysis—Effector Prediction and Genome Evolution

Robert A. Syme¹, Kar-Chun Tan¹, Kasia Rybak¹, Timothy L. Friesen², Bruce A. McDonald³, Richard P. Oliver¹, and James K. Hane¹,4,*

¹Centre for Crop & Disease Management, School of Molecular & Life Sciences, Curtin University, Bentley, Western Australia, Australia
²Cereal Crops Research Unit, USDA-ARS Red River Valley Agricultural Research Center, Fargo, North Dakota
³Plant Pathology Group, Institute of Integrative Biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland
⁴Curtin Institute for Computation, Curtin University, Bentley, Western Australia, Australia

*Corresponding author: E-mail: james.hane@curtin.edu.au.

Accepted: September 3, 2018

Data deposition: This project has been deposited at GenBank under accessions PRJNA476481, SRX4494197-SRX4494226 and QVWA00000000-QVXD00000000

Abstract

We report a fungal pan-genome study involving Parastagonospora spp., including 21 isolates of the wheat (Triticum aestivum) pathogen Parastagonospora nodorum, 10 of the grass-infecting Parastagonospora avenae, and 2 of a closely related undefined sister species. We observed substantial variation in the distribution of polymorphisms across the pan-genome, including repeat-induced point mutations, diversifying selection and gene gains and losses. We also discovered chromosome-scale inter and intraspecific presence/absence variation of some sequences, suggesting the occurrence of one or more accessory chromosomes or regions that may play a role in host–pathogen interactions. The presence of known pathogenicity effector loci SnToxA, SnTox1, and SnTox3 varied substantially among isolates. Three P. nodorum isolates lacked functional versions for all three loci, whereas three P. avenae isolates carried one or both of the SnTox1 and SnTox3 genes, indicating previously unrecognized potential for discovering additional effectors in the P. nodorum-wheat pathosystem. We utilized the pan-genomic comparative analysis to improve the prediction of pathogenicity effector candidates, recovering the three confirmed effectors among our top-ranked candidates. We propose applying this pan-genomic approach to identify the effector repertoire involved in other host–microbe interactions involving necrotrophic pathogens in the Pezizomycotina.

Key words: Parastagonospora nodorum, Pan-genome, plant pathogen, crop disease, host–microbe interactions.

Introduction

The Parastagonospora Pathosystems

Parastagonospora (teleomorph: Phaeosphaeria) nodorum (Berk.) is an economically important necrotrophic fungal pathogen that causes septoria nodorum blotch (SNB) in wheat (Triticum aestivum) (Solomon et al. 2006) and is also a model organism for the fungal order Pleosporales and for necrotrophic phytopathogenicity (Oliver and Solomon 2010; Tan et al. 2010; Oliver et al. 2012). Significant experimental resources are available for P. nodorum, including a high-quality genome assembly of the reference strain SN15 (Hane et al. 2007; Syme et al. 2016), microarray analyses of gene expression (Ipcho et al. 2012), proteomics and proteogenomics, metabolomic profiling, and comparative genomics versus two contrasting P. nodorum strains: Sn4 and Sn79-1087 (Syme et al. 2013; Richards et al. 2018). SN15 and Sn4 are highly aggressive isolates on wheat, whereas Sn79-1087 (hereafter referred to as Sn79) was isolated from the wild grass Agropyron, is avirulent on wheat, and serves as a useful negative control for comparative genomics in a disease context (Friesen et al. 2006; Richards et al. 2018).

In this study we resequenced the genomes of 18 new strains of P. nodorum as well as 10 strains representing closely related species within the Parastagonospora genus (table 1). Parastagonospora avenae is a species associated with SNB-like symptoms in various Poaceae spp. (Quaedvlieg et al. 2013). P. avenae was further subdivided into two formae

GBE

Genome Biol. Evol. 10(9):2443–2457. doi:10.1093/gbe/evy192 Advance Access publication September 4, 2018 2443
### Table 1
Summary of Source Material and Resequencing Data for Strains of (A) *P. nodorum*, (B) *P. avenae*, and (C) the P2 clade

| Isolate ID | Source | Collection Year | Total Length of Error-corrected Reads (Mb) | Estimated Genome Coverage (X) | Isolation Source | Contributor |
|------------|--------|-----------------|---------------------------------------------|-------------------------------|-----------------|-------------|
| **(A) *P. nodorum* strains** | | | | | | |
| B2.1b | Iran | 2005 | 686.1 | 9.2 | T. aestivum | Mohammad Razavi, Iranian Research Institute of Plant Protection |
| C1.2a | Iran | 2010 | 886.1 | 11.9 | | | |
| IR10_9.1a | Finland | unknown | 528.4 | 7.1 | | | |
| FIN-2 | Finland | unknown | 1,998.7 | 26.9 | | Andrea Ficke, Nibio, As, Norway |
| SWE-3 | Sweden | 2010 | 1,517.8 | 20.4 | | | |
| Sn Cp2052 | | 2011 | 1,031.9 | 13.9 | | | |
| BRSn9870 | Brazil | | 3,316.9 | 44.6 | | Flavio Santana, EMBRAPA |
| Sn99CH 1A7a | Switzerland | 1999 | 3,888.2 | 52.3 | | Bruce McDonald, ETH Zurich |
| SnCh01 40a | China | 2001 | 2,796.4 | 37.6 | | R. Wu, Louyuan, Fujian Province |
| SnSA95.103 | South Africa | 1994 | 1,019 | 13.7 | | | |
| AR1-1 | Arkansas, USA | 2011 | 1,986.2 | 26.7 | | | |
| GA9-1 | Georgia, USA | 1994 | 4,296.5 | 57.8 | | | |
| MD4-1 | Maryland, USA | 2012 | 4,325.8 | 58.2 | | | |
| VA 5-2 | Virginia, USA | | 2,288.3 | 30.8 | | | |
| OH03 Sn-1501 | Ohio, USA | 2003 | 2,229.8 | 30 | | Pat Lipps, Ohio State University |
| SNOV92X D1.3 | Texas, USA | 1992 | 2,524.6 | 34 | | Bruce McDonald, Texas A&M University |
| SnOre11-1 | Oregon, USA | 2011 | 4,763.4 | 64.1 | | | |
| WAC8410 | Australia | 2010 | 6,032.4 | 81.2 | | | |
| **(B) *P. avenae* strains** | | | | | | |
| (i) *P. avenae* f. sp. *triticea* 1 | | | | | | |
| IR10_5.2b | Iran | 2010 | 850.5 | 11.4 | T. aestivum | Mohammad Razavi, Iranian Research Institute of Plant Protection |
| Hartney99 | Canada | 2005 | 1,132.5 | 15.2 | T. aestivum (spring wheat) | | |
| Jansen #4_55 | | | 480.1 | 6.5 | | | |
| (ii) *P. avenae* f. sp. *triticea* 4 | | | | | | |
| SN11R_2_2_1.1 | Iran | 2011 | 1,415.3 | 19 | Dactylis glomerata | Mohammad Razavi, Iranian Research Institute of Plant Protection |
| SN11R_7_2.3 | | | | | | |
| **(C) P2 strains** | | | | | | |
| (McDonald et al. 2012) | | | | | | |
| A1 3.1a | Iran | 2005 | 1,813.1 | 24.4 | T. aestivum | Mohammad Razavi, Iranian Research Institute of Plant Protection |
| H6.2b | | 2005 | 3,030 | 40.8 | | | |
speciales. P. avenae f. sp. avenaria (Paa) infects oats (Avena spp.) and P. avenae f. sp. triticea (Pat) infects wheat and some other grasses (Cunfer 2000; Ueng et al. 2003; Malkus et al. 2005). Restriction fragment length polymorphism patterns were used to further differentiate Pat strains into distinct subgroups named Pat1-Pat6 (Ueng and Chen 1994; Ueng et al. 1995; Ueng et al. 1998). Two isolates of a sister species, provisionally named P2 (Phaeosphaeria 2) were collected from Iranian wheat fields. The placement of P2 is not yet clear, but sequences of the β-tubulin and β-xylanase loci were similar to P. nodorum and P. avenae (McDonald et al. 2012).

Many of the recent studies of Parastagonospora spp. are oriented around an important class of genes encoding effector molecules that interact with the host to determine the outcome of specific host-pathogen interactions (Vleeshouwers and Oliver 2014). A class of effectors called necrotrophic effectors (NEs) interacts with host dominant susceptibility genes to form an inverse gene-for-gene interaction (Oliver and Solomon 2010; Tan et al. 2010). A compatible interaction results in plant cell death and subsequently, infection. Parastagonospora spp. were already shown to produce several NEs (Friesen et al. 2007), which are thought to maximize the likelihood of interacting with a corresponding susceptibility protein in the host. So far, three well-characterized NE genes have been identified in P. nodorum: SnToxA (SNOG_16571) (Friesen et al. 2006), SnTox1 (SNOG_20078) (Liu et al. 2004; Liu et al. 2012), and SnTox3 (SNOG_08981) (Friesen et al. 2008; Liu et al. 2009). These three effectors are already used in breeding programs to accelerate development of disease-resistant cereal cultivars (Vleeshouwers and Oliver 2014), but several NE loci have not yet been identified to the gene level, including SnTox2 (Friesen et al. 2007), SnTox4 (Abeysekara et al. 2009), SnTox5 (Friesen et al. 2012), SnTox6 (Gao et al. 2015), and SnTox7 (Shi et al. 2015). Although continued laboratory testing may yield new effectors, further advances in genome sequencing technologies and bioinformatic methods may also improve effector discovery (Jones et al. 2018). This study provides enhancements to the P. nodorum SN15 reference strain assembly and its gene annotations, but also explores features of the Parastagonospora fungal genomes that are relevant for effector discovery, including repeat-induced point mutation (RIP), presence–absence variation (PAV), and diversifying selection.

RIP is a fungal-specific form of mutation that targets repetitive sequences and introduces cytosine to thymine (C → T) transitions, or the reverse complement G → A. In the filamentous Ascomycota (syn. Pezizomycotina) where RIP is commonly observed (Testa et al. 2016), there is a strong bias for mutations at cytosine bases adjacent to adenine (CpA → TpA). RIP provides a mechanism of genome defense against transposon invasion, by disabling transposable elements through introduction of premature stop codons into their open reading frames and/or through silencing of the RIP-mutated sequence through further DNA methylation (Galagan and Selker 2004; Hane and Oliver 2008; Clutterbuck 2011; Hane 2015; Hane et al. 2015). RIP has also been linked to mutation of avirulence effector genes AvrLm1, AvrLm6, and AvrLm4-7 in Leptosphaeria maculans through leakage into nonrepetitive regions flanking repeats (Fudal et al. 2009; Van de Wouw et al. 2010). Fungal genes most likely to be affected by RIP leakage can be identified by annotation of AT-rich regions with OcculterCut (Testa et al. 2016).

Recent resequencing studies have repeatedly shown that PAV in gene content is common in fungi (McDonald et al. 2013; Gao et al. 2015; Golicz et al. 2015). PAV patterns have been observed at both the gene cluster level (Plissonneau et al. 2016) and at whole (or partial) chromosome level (Ma et al. 2010; Goodwin et al. 2011), and may indicate variable effector loci when applied across multiple isolates of a single species with a range of virulence phenotypes. Sectional absences of small groups of genes were previously reported between the reference strain SN15 and other strains of P. nodorum (Syme et al. 2013). PAVs related to known effectors vary in length, with SnTox1 and SnTox3 absent from the wheat-avirulent strain SN79 in small 2 and 4 kb stretches, respectively, whereas SnTox4 is part of a much larger 72 kb absence in SN79 (Syme et al. 2013). The PAV pattern of known effectors also varies in field populations of Parastagonospora spp. (fig. 1), which may indicate multiple, independent horizontal gene transfer (HGT) events (McDonald et al. 2013). Notably, there does not appear to be a significant fitness penalty incurred by the pathogen harboring an effector when growing on a host that lacks the corresponding sensitivity gene (McDonald et al. 2013). Genes within repeat-rich regions may be more prone to loss due to mesosyntenic recombination (Hane et al. 2011) or breakage fusion bridge cycles (Bertazzoni et al. 2018).

The genomes of some pathogenic species are compartmentalized into regions with “two speeds” of evolution: “core” gene content—which tends to be well-conserved among strains and under purifying selection, and “accessory” gene content—which typically is repeat-rich and exhibits both PAV and evidence for diversifying selection. For some species, such as L. maculans, these variable regions are interspersed throughout the genome. In other species, accessory and core gene contents are divided among separate chromosomes (Bertazzoni et al. 2018). Fungal accessory (syn. supernumerary or dispensable) chromosomes have been observed to be gene sparse, repeat-rich, and exhibit higher rates of mutation, positive selection and pathogenicity-associated loci relative to the “core” chromosomes (Bertazzoni et al. 2018). The P. nodorum isolates in this study may also contain accessory sequences, as wheat- and barley-infecting isolates have been previously reported to possess an accessory chromosome of approximately 400 kb (Zolan 1995). Furthermore, P. nodorum SN15 scaffold_46 was previously predicted to be dispensable based on sharing common characteristics with Z. tritici accessory chromosomes, including low GC content, high repeat content, low gene density and a low percentage of
predicted genes with conserved functional domains (Ohm et al. 2012).

Recent advances in understanding fungal genome evolution coupled with improved criteria for identifying candidate effector genes provide new opportunities to understand the role of intraspecies genome diversity in the context of pathogenicity. Here, we compare 19 isolates of *P. nodorum*, 10 isolates of *P. avenae*, and 2 isolates of the closely related sister group *P*. materials and Methods

Strain Sampling, DNA Extraction and Sequencing

Illumina paired-end libraries were constructed for each haploid strain. *P. nodorum* WAC8410 was sequenced from a TruSeq 500 bp library on an Illumina 2000 to produce 150 bp paired-end reads. All other strains were sequenced from 300 bp NexteraXT libraries on an Illumina 2500 multiplexed over two lanes.

Reference-Alignment and de novo Assembly of Strain Sequences

Raw Illumina reads were filtered for PCR duplicates, then trimmed using cutadapt v1.7.1 (Martin 2011), removing the adapter sequences, bases with quality scores less than 25, and any reads shorter than 50 bp. *P. nodorum* strain SN15 (Hane et al. 2007) was used as the reference against which each of the newly sequenced strains was compared. The annotated gene set of SN15 has been improved with transcriptomic and proteomic data, and manual curation of gene models (Syme et al. 2016). Trimmed reads were aligned to the reference genome using bowtie2 (“very-sensitive”). Trimmed reads were also assembled de novo with SPAdes v.3.5.0 (Bankevich et al. 2012) (kmers 21, 33, 55, and 77) using mismatch and short indel correction via BWA mapping (Li 2013). Assembly quality was assessed with QUAST v2.3 (Gurevich et al. 2013). Genome assemblies were aligned to the reference with MUMmer v3.0 (Kurtz et al. 2004) and repetitive matches were filtered with delta-filter (–r and –q). SNP and indel variants from genome alignments were determined with MUMmer (showsnps). PAV profiles were generated by calculating the coverage of each of the SN15 reference scaffolds by nucmer matches to each isolate assembly using the genocov function of BEDTools (Quinlan and Hall 2010). Homologs of known effectors were extracted from orthologous...
clusters. Each effector absence was manually confirmed by blasting the reference effector against the new strain’s genome assembly.

**Genome Annotation**

Manually curated annotations from the reference genome SN15 (Syme et al. 2016) were used to train CodingQuarry (Testa et al. 2015) and these parameters were applied to each of the alternate strains. A database of repeats was generated using RepeatModeler v1.0.8 (Smit and Hubley 2010) augmented with full copies of the known *P. nodorum* repeats Molly (AJ488502.1), Pixie (AJ488503.1), and Elsa (AJ277966.1). The RepeatModeler repeats were combined with known “DeRIPped” (predicted pre-RIP consensus) (Hane and Oliver 2010) and repbase (Jurka et al. 2005) repeats using RepeatMasker v4.0.5 (Smit et al. 2015). A final annotation set for each alternate strain (supplementary file S1, Supplementary Material online) was generated using Augustus v3.3 (Stanke et al. 2006) using annotation hints provided CodingQuarry annotations, exonerate v2.2.0 protein matches (Slater and Birney 2005) to the SN15 predicted proteome and from

---

**Table 2**

Summary of Genome Assembly Metrics for Resequenced Strains of (A) *P. nodorum*, (B) *P. avenae*, and (C) the P2 clade

| Isolate ID   | No Scaffolds | Largest Scaffold (kb) | Total Length (Mb) | N50 (kb) | Whole SN15 Gene Count by QUAST | Partial SN15 Gene Count by QUAST |
|--------------|--------------|-----------------------|-------------------|----------|-------------------------------|----------------------------------|
| **(A) *P. nodorum* strains** | | | | | | |
| B2.1b | 2,906 | 386.4 | 37.38 | 60.3 | 12,628 | 784 |
| Cl2a | 1,557 | 325.3 | 37.43 | 80.4 | 12,751 | 649 |
| IR10.9.1a | 3,673 | 140.6 | 37.28 | 20.7 | 11,442 | 1,920 |
| FIN-2 | 1,381 | 451.6 | 38.41 | 117.9 | 12,952 | 479 |
| SWE-3 | 1,714 | 335.5 | 37.85 | 58.8 | 12,734 | 727 |
| Sn Cp2052 | 3,026 | 190.2 | 37.32 | 38.2 | 12,310 | 1,148 |
| BRSn9870 | 4,911 | 309.9 | 41.23 | 52.4 | 12,342 | 1,148 |
| Sn99CH 1A7a | 853 | 521 | 37.9 | 180.7 | 13,062 | 361 |
| SnChio1 40a | 779 | 1,268.10 | 37.88 | 206.3 | 13,118 | 332 |
| SnSA95.103 | 11,772 | 48 | 49.94 | 6.5 | 9,545 | 3,882 |
| AR1-1 | 882 | 748.5 | 36.61 | 135.6 | 12,999 | 412 |
| GA9-1 | 664 | 938.6 | 36.53 | 215.3 | 13,074 | 356 |
| MD4-1 | 701 | 599.9 | 36.53 | 191 | 13,045 | 384 |
| VA 5-2 | 1,115 | 485.6 | 36.48 | 89.1 | 12,787 | 606 |
| OH03 Sn-1501 | 1,281 | 349.9 | 37.1 | 85 | 12,844 | 577 |
| SN9V92X D1.3 | 785 | 524.1 | 36.66 | 145.6 | 13,009 | 442 |
| SnOre11-1 | 748 | 875.4 | 37.42 | 249.6 | 13,103 | 352 |
| WAC8410 | 384 | 1,060.50 | 40.27 | 316.8 | 13,223 | 277 |
| **(B) *P. avenae* strains** | | | | | | |
| (i) *P. avenae* f. sp. *triticea* | | | | | | |
| IR10_5.2b | 1,681 | 267 | 35.51 | 57.3 | 18 | 14 |
| Hartney99 | 3,381 | 124.3 | 36.58 | 27.6 | 47 | 20 |
| Jansen 4.55 | 10,109 | 83.1 | 32.06 | 4.3 | 20 | 166 |
| (ii) *P. avenae* f. sp. *triticea* | | | | | | |
| SN11IR_2_1.1 | 5,762 | 168.2 | 41.54 | 38.6 | 12 | 15 |
| (iii) *P. avenae* f. sp. *triticea* | | | | | | |
| B2-4841 | 2,444 | 218.3 | 38.53 | 50.5 | 21 | 13 |
| B3-6011-2 | 2,367 | 193.8 | 37.52 | 43.9 | 21 | 13 |
| (iv) *P. avenae* f. sp. *triticea* | | | | | | |
| SN11IR_6_1.1 | 1,174 | 737.1 | 33.51 | 102.8 | 3 | 7 |
| SN11IR_7_2.3 | 2,215 | 244.5 | 33.6 | 44.8 | 6 | 12 |
| (v) *P. avenae* f. sp. *Avenaria* | | | | | | |
| Mt. Baker | 8,309 | 38.5 | 34.14 | 6.2 | 15 | 81 |
| s258 | 4,090 | 183.1 | 39.49 | 32.8 | 16 | 21 |
| **(C) P2 strains** | | | | | | |
| H6.2b | 1,613 | 411.9 | 36.88 | 74.6 | 2 | 6 |
| A1 3.1a | 1,764 | 419.9 | 39.05 | 68.3 | 3 | 6 |

Note.—*P. avenae* and P2 strains show a low number of *P. nodorum* gene matches from QUAST due to dissimilarities in the coding sequence relative to the *P. nodorum* SN15 reference.
RepeatMasker matches described above. Secretion signals were detected using SignalP v4.1 (Bendtsen et al. 2004) and transmembrane domains by TMHMM v2.0 (Krogh et al. 2001).

Secondary metabolite clusters were predicted in nonreference strains by antismash 2.1.1 (Medema et al. 2011). Orthofinder v2.2.3 (Emms and Kelly 2015) using Diamond v0.9.21
For distance estimation was used to predict clusters of orthologous proteins and to generate a phylogeny of the isolates used in this study, using the closely related species *Pyrenophora tritici-repentis* as an outgroup (Moolhuijzen et al. 2018). In the case of known effector loci SnToxA, SnTox1 and SnTox3, their ortholog cluster membership was manually inspected by tblastn (Altschul et al. 1990) (e-value cutoff $1 \times 10^{-5}$) queries of their translated sequences versus each of the alternate strain de novo assemblies.

**Identification of Genes under Diversifying Selection**

Coding sequences from all strains that were orthologous to protein-coding genes from the reference strain were extracted, translated, and aligned using ClustalW (Larkin et al. 2007). Protein truncations due to incorrect annotation in a novel strain can limit detection of diversifying selection, so only codons present in all strains were considered. Short proteins with lengths more than 1 standard deviation from the mean were excluded from these alignments to reduce the frequency of false-positive results.

Codon alignments were generated with PAL2NAL v14 (Suyama et al. 2006). The M1a and M2a site models were applied to codon-aligned transcripts of orthologous proteins to generate a maximum likelihood (ML) estimation of $\omega$. The $H_0$ model (PAML model M1a) confines codon membership to one of two classes where $\omega < 1$ (purifying selection) or $\omega = 1$ (neutral/drift). The $H_1$ model (PAML model M2a) extends $H_0$ to allow codon membership to a third possible class where $\omega > 1$ (diversifying selection). Loci with sites under diversifying selection were identified where the $\chi^2$-distributed likelihood ratio of the two models exceeded the 1% significance level (2 degrees of freedom). Local patterns of selection were identified by stepping a 100 kbp window over the reference assembly in 1 kbp increments and counting the number of transcripts under diversifying selection as a percentage of the total number of transcripts in each window.

**Prediction of Effector Candidates**

For each reference transcript sequence, a number of tests were applied and a cumulative score was generated based
on the number of passed tests. Protein scores were increased if they had a molecular mass less than 30 kDa, had a cysteine percentage greater than 4%, were longer than 200 bp, did not have tblastn hits to the wheat-avirulent Sn79 genome assembly (e-value ≤ 1 × 10⁻30), were in regions of low gene density (no genes predicted within the 2 kb region(s) up/downstream), were predicted to be secreted by SignalP v4.1 (Bendtsen et al. 2004), were predicted to be under positive diversifying selection pressure as described above, were not part of the core proteome, or were not predicted to encode a transmembrane domain by TMHMM (Krogh et al. 2001), were less than 5 kb from the nearest AT-rich region as predicted by OcculterCut, or had an EffectorP score greater than 0.9.

**Table 3**

Summary of Protein Conservation Across the *P. nodorum* and *P. avenae* Strains

| Reference Protein Set | 13,836 proteins |
|-----------------------|-----------------|
| Core Phaeosphaeria protein set | |
| Missing from 0 strains | 8,660 clusters |
| Missing from at most 1 strain | 9,921 clusters |
| Missing from at most 2 strains | 10,798 clusters |
| Core *P. nodorum* protein set | |
| Missing from 0 *P. nodorum* strains | 11,366 clusters (11,821 SN15 proteins) |
| Missing from at most 1 *P. nodorum* strain | 12,473 clusters (12,877 SN15 proteins) |
| Missing from at most 2 *P. nodorum* strains | 13,049 clusters (13,139 SN15 proteins) |
| Strain-specific protein set | |
| Observed in only 1 strain | 3,995 clusters (108 SN15 proteins) |
| Observed in at most 2 strains | 5,438 clusters (240 SN15 proteins) |
| Observed in at most 3 strains | 6,229 clusters (291 SN15 proteins) |
| Dispensable protein set (effector-containing set) | |
| Observed in between 4 and 30 strains (inclusive) | 6,620 clusters (321 SN15 proteins) |
| *P. nodorum*-specific proteins | |
| Present in all *nodorum*, absent from all *P. avenae* | 216 |
| *P. avenae*-specific proteins | |
| Present in all *avenae*, absent from all *P. nodorum* | 44 |

**Results**

Pan-Genome Resources Generated for Multiple Isolates of *P. nodorum*, *P. avenae* and P2

The *Parastagonospora* strains originated from a broad geographical range with a focus on the Fertile Crescent and the United States (table 1 and fig. 1) and included three previously sequenced *P. nodorum* strains (Syme et al. 2016; Richards et al. 2018), 18 newly sequenced *P. nodorum* strains, 10 newly sequenced *P. avenae* strains, and 2 newly sequenced strains of the closely related P2 group (McDonald et al. 2012) (BioProject: PRJNA476481). The estimated genome coverage for each strain, relative to the SN15 reference assembly, ranged between 7–64X for strains sequenced using the NexteraXT libraries and 81X for the WAC8410 isolate sequenced using a separate TruSeq library (table 1). Total genome assembly length ranged from 33.5 to 49.9 Mb (table 2).

**SnToxA, SnTox1, and SnTox3 Effector Loci Vary in Their Distribution Across Both Taxonomic Groups and Geographic Locations**

The ortholog cluster containing the *SnToxA* effector gene was found in 10 *P. nodorum* strains (48%) but no *P. avenae* strains. Intact *SnTox1* gene sequences were detected in 18 *P. nodorum* strains (86%) and 2 *P. avenae* strains (17%). The *SnTox3* gene was detected in 11 *P. nodorum* strains (52%) and the 2 *P. avenae* Pat5 strains (figs. 1 and 2).

Presence/Absence Patterns Across the *Parastagonospora* Pan-Genome Indicate Putative Lineage-Specific Accessory Sequences

There were differing outcomes for sectional absence based on the read-mapping methods employed in previous studies (Croll and McDonald 2012; Croll et al. 2013; McDonald et al. 2015; Syme et al. 2016) compared with de novo assembly and subsequent alignment via nucmer used here. For comparisons between *P. nodorum* strains and the SN15 reference, the read-mapping method was able to genotype 90.3% of the genome. In contrast, the nucmer method found fewer sectional absences and was able to identify variants over 93.5% of the genome. In more distant comparisons of *P. avenae* strains to the SN15 reference, read-mapping was able to genotype only 16.2% of the genome and nucmer was able to find variants over 61.9% of the genome.

Of the 51 *P. nodorum* SN15 reference scaffolds that contained genes, most had consistent nucmer match coverage by sequences of the resequenced strains. Scaffolds 44, 45, 51, 57, 69, 97, 99, and 101 had very low coverage by alignment of *P. avenae* and P2 isolates (fig. 3). These scaffolds are composed largely of repeats, except scaffolds 44 and 45 which respectively encode 75 and 61 genes and contain only 1.9% and 2.4% repetitive sequence. Scaffolds 44, 45 also had a high percentage of genes under diversifying selection (29.3% and 23.0%). The wheat-avirulent *P. nodorum* strain SN79 also covered scaffolds 44 and 45 over 6.7% and 5.0% of...
Patterns of Strain-Specificity and Positive Selection Across the Pan-Genome Refines Effector Candidate Predictions

The distribution of conserved proteins between the P. avenae and P. nodorum strains shows most proteins are either strain-specific or are well conserved, but known effectors are present in the sparsely populated middle-ground (fig. 4). Of the 21,470 orthologous protein groups, 6,229 (29.0%) were observed in fewer than four strains, 12,473 (58.1%) were missing from at most one strain, and 6,620 (30.8%) were between the two extremes (table 3 and fig. 4). P. nodorum strain WAC8410 has 184 strain-specific loci (supplementary file S1 and table S2, Supplementary Material online). This strain WAC8410 has 184 strain-specific loci (supplementary be between the two extremes (table 3 and fig. 4). P. nodorum strain WAC8410 has 184 strain-specific loci (supplementary file S1 and table S2, Supplementary Material online). This strain was sequenced at higher coverage (table 1) and is a more complete assembly (table 2) than the other strains, enabling a more detailed study of its strain-specific loci. The mean intergenic flanking distance for all WAC8410 genes is 1,162 bp. The mean intergenic flanking distance for the strain-specific subset of WAC8410 genes is 6,558 bp (fig. 5). WAC8410 scaffolds 50 and 55 are rich in strain-specific genes, with 18 of 20 and 11 of 23, respectively. At least 8 of the 18 strain-specific genes on scaffold 55 appear to be pseudogenes with high rates of mutation and premature stop codons. Included in the scaffold 55 strain-specific set are pseudogenes which shows similarity to the P. nodorum PhnP2 transcription factor (Rybak et al. 2017), an indole-diterpene biosynthesis protein, and a pseudogene which shows similarity to SNOG_08983 which, in SN15, is notably near to the SnTox3 locus. Effector candidates were predicted from the SN15 reference protein set by using a series of criteria based on the expected characteristics of NEs (table 4). Scores were calculated for 13,949 SN15 proteins, of which: 616 were cysteine-rich; 451 were associated with AT-rich regions; 798 were absent from Sn79; 2,414 resided in regions of low gene density; 1,475 were predicted as secreted; and 945 were under positive selection. Effector candidate scores are presented in full in supplementary table S3, Supplementary Material online, and 63 candidates with scores of 5 and above are presented in table 5. Known effector genes SnTox4, SnTox1, and SnTox3 all scored highly using this system (table 5). Of the P. avenae isolates sequenced, only

Their lengths, respectively, compared with 94.4% and 96.3% coverage by the other P. nodorum strains.

the wheat-avirulent Pat5 genomes (McDonald et al. 2012) contained putative SnTox3 orthologs (fig. 1). Interestingly, the genomic context of this gene in P. avenae is different compared with the SnTox3 positive P. nodorum strains. The putative SnTox3 ortholog in P. avenae Pat5 is in the position of a Zn-dependent oxidoreductase in P. nodorum and the other P. avenae genomes (fig. 5).
Table 5
Top Effector Candidates with Scores ≥5

| Locus       | Predicted Secreted | Absent in SN79 | <1 Gene/2 kb | ≤30 kDa Positive Selection | ≥4% Cys | AT-Rich Regions | Effector PS Score ≥0.9 | Candidate Score ≥5 |
|-------------|---------------------|----------------|-------------|-----------------------------|---------|----------------|-----------------------|-------------------|
| SNOG_20078 (SnTox1) | ✓                  | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_01124 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_11452 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_11453 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_30077 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_30343 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_30828 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_16571 (SnToxA) | ✓                  | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_08981 (SnTox3) | ✓                  |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_01097 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_07039 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_12811 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_16520 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_20100 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_30802 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_00726 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_03114 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_04279 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_04353 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_05030 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_06202 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_06459 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_07292 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_08206 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_08469 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_08606 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_09147 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_09446 | ✓                   | ✓              | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_09672 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_10135 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_10664 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_11632 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_12350 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_12382 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_12564 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_12748 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_13126 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_13993 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_14135 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_14618 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_14826 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_15074 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_15150 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_15417 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_15989 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_16091 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_16131 | ✓                   |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |
| SNOG_16166 (scaffold 44) |                  |                  | ✓           | ✓                           | ✓       | ✓              | ✓                     | ✓                 |

(continued)
Interestingly, 4 of the top 63 effector nate strain (Syme et al. 2013). Regions that are highly differ-
ted relative to the reference genome can prevent
reads from mapping to the reference, inflating the count
of genes absent in the alternate strain. Comparisons
based on de novo assemblies of the alternate strain pro-
duced a greater coverage of the reference genome for
both P. nodorum and P. avenae. Figure 6 illustrates this
using a region in the reference genome where reads from
P. nodorum IR10_2.1a failed to map. The region without
mapped reads covers 20 kb and includes eight reference
genes. A protocol that calculates gene absence from map-
ing data alone would describe this as an 8-gene sectional
absence. However, using nucmer to align de novo assem-led sequences of this strain to the reference reveals that
the absence is only 8 kb and that only two genes are ab-
sent from the alternate strain. The surrounding regions
are sufficiently variable to prevent the read mapping algo-
rithms from mapping reads and providing coverage. In
variable genomic regions that exceed the tolerances of
read mapping algorithms, a hybrid mapping/assembly ap-
proach to variant detection is likely to provide a more
complete picture of sequence differences.

The Parastagonospora spp. pan-genome data set made it
possible to assess positive selection for almost every locus of
the reference isolate SN15. The frequency distribution of
genes under diversifying selection allowed us to divide the
genome into three categories: (1) a background rate of 5–
10% genes under diversifying selection, which represented
the majority of the genome; (2) large regions where 20–30%
of genes are under diversifying selection. Examples include
the putative accessory sequences on scaffolds 44 and 45;
and (3) small islands of genes adjacent to repetitive regions
under diversifying selection, such as scaffolds 7, 15, and 20
(supplementary tables S3 and S5, and fig. S4, Supplementary
Material online).

We hypothesize that these categories may relate to the
biological function of the genes they contain. The first cate-
gory is likely to contain the bulk of the stable genome and the
housekeeping genes undergoing long term and continuous
adaptation to the environment. The second category repre-
sents accessory genomic regions involved in rapid changes
such as adaptation to new hosts species or genotypes.
Genes contained in regions of the third category may repre-
sent coordinately regulated gene clusters such as those in-
volved in secondary metabolism. These category 3 regions

Table 5 Continued

| Locus            | Predicted Secreted | Absent in SN79 | <1 Gene/2 kb | ≤30 kDa | Positive Selection ≥4% Cys | AT-Rich Regions | Effector P Score ≥0.9 | Candidate Score ≥5 |
|------------------|--------------------|----------------|-------------|---------|----------------------------|-----------------|----------------------|------------------|
| SNOG_16226 (scaffold 44) | ✓                 | ✓              | -           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_16236 (scaffold 44)  | ✓                 | ✓              | -           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_16237 (scaffold 44)  | ✓                 | ✓              | -           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_16270 (scaffold 45) | -                 | ✓              | ✓           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_16345 (scaffold 45) | -                 | ✓              | ✓           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_20011         | ✓                 | ✓              | -           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_30026         | ✓                 | ✓              | -           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_30316         | ✓                 | ✓              | -           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_30334         | ✓                 | -              | ✓           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_30645         | ✓                 | -              | ✓           | ✓       | -                          | -               | ✓                    | $5               |
| SNOG_30701         | ✓                 | ✓              | -           | ✓       | -                          | -               | ✓                    | $5               |

et al. 2011), Zymoseptoria tritici (Croll et al. 2015; Goodwin et al. 2011), and Fusarium oxysporum (Ma et al. 2010). We speculate that these scaffolds may comprise one or more
accessory chromosomes or regions (Covert 1998; Croll and
McDonald 2012). Their absence from Sn79 demonstrates
that the genes on these scaffolds are not strictly required
for survival, and their retention in more virulent strains sug-
gests that they may confer some advantages in some host
environments. The strong positive selection observed in SN15
scaffolds 44 and 45 also suggests that the localized accumu-
lation of mutations in these putative accessory regions may
promote genetic innovation (Croll and McDonald 2012; Oliver
2012; Croll et al. 2013). Interestingly, 4 of the top 63 effector
candidate loci (score ≥ 5, table 5) resided on SN15 scaffold 44
(SNOG_16166, SNOG_16226, SNOG_16236, and
SNOG_16237) and 2 more (SNOG_16270 and
SNOG_16345) resided on SN15 scaffold 45 (supplementary
file S1 and table S3, Supplementary Material online). Scaffolds
50 and 55 in isolate WAC8410 also exhibited the low gene
density typical of accessory chromosomes (Covert 1998;
Goodwin et al. 2011), as well as an extremely high
frequency of pseudogenes with identifiable functional paralogs.
Collectively, these anomalous scaffolds share many of the
features typically associated with accessory chromosomes
of other plant pathogens (Goodwin et al. 2011; Croll et al.
2015).

By surveying the SNP distribution and density, we identified
an oversight associated with previously used short-read se-
quencing approaches. Previous genome comparisons be-
tween P. nodorum species used the depth of reads mapped
to the reference genome to infer gene absence in the alter-
ate strain (Syme et al. 2013). Regions that are highly differ-
entiated relative to the reference genome can prevent
reads from mapping to the reference, inflating the count
of genes absent in the alternate strain. Comparisons
based on de novo assemblies of the alternate strain pro-
duced a greater coverage of the reference genome for
both P. nodorum and P. avenae. Figure 6 illustrates this

Genome Biol. Evol. 10(9):2443–2457 doi:10.1093/gbe/evy192 Advance Access publication September 4, 2018 2453
are adjacent to repeat-rich stretches of the genome, which in other species are associated with secondary metabolite synthesis gene clusters (Dallery et al. 2017). RIP is known to encroach from repetitive regions into nearby single-copy genes, in some cases disabling recognized effector genes (Fudal et al. 2009; Rouxel et al. 2011). In the case of genes encoding avirulence effectors, mutation can be beneficial to the pathogen by enabling evasion of recognition on a host carrying the corresponding resistance gene. Islands of positive selection were observed to often be adjacent to AT-rich regions (Testa et al. 2016), which suggests there may be an association between RIP and effector loci in \textit{P. nodorum}.

The known \textit{P. nodorum} effector genes are all positioned near repetitive sequences (Syme et al. 2013; Kellner et al. 2014). It has been suggested that repetitive elements provide a mechanism for effector mobilization within genomes and HGT between species, while also increasing diversity at these loci (Croll and McDonald 2012; Oliver 2012; Croll et al. 2013). Notably, a putative SnTox3 ortholog was observed in both \textit{P. avenae} Pat5 isolates, within a gene-dense region that is otherwise syntenic to the other strains sequenced and shows no sign of nearby transposon activity (fig. 5). The evolutionary history of these homologs at two different loci is unclear. Possible explanations include independent acquisition of the gene by \textit{P. nodorum} and Pat5 ancestors, or lateral transfer between these taxa. Another possibility is that the location in Pat5 is the ancestral version, and subsequent translocation in \textit{P. nodorum} adjacent to a repeat-rich region may provide an adaptive advantage in the form of RIP-mediated accelerated mutation rates (Fudal et al. 2009; Van De Wouw and Howlett 2011; Testa et al. 2016). The absence of any isolate with a copy in both locations likely indicates either direct
translocation or transduplication with subsequent loss of the original paralog, likely due to selection pressures imposed by RIP on duplicated sequences (Galagan and Selker 2004; Hane and Oliver 2008).

The addition of new genome assemblies allowed us to expand the set of criteria used to identify candidate effector genes. The presence/absence allele frequency in a P. nodorum population differs for each effector, and was hypothesized to reflect the prevalence of each effector’s susceptibility gene in the corresponding host population (McDonald et al. 2013). SnTox4, SnTox7 and SnTox8 all showed presence/absence polymorphisms, and each were individually observed in 50–85% of the P. nodorum isolates studied (fig. 1). A more accurate determination of core and strain-specific genes (fig. 3 and table 3) allows us to better identify genes that are neither perfectly conserved nor infrequent. Based on updated criteria for ranking effector candidate loci, SnTox1 is among the eight equally top-ranked candidates and SnTox3 and SnTox4 are among the nine 2nd-ranked candidates (table S5 and supplementary table S3, Supplementary Material online).

The 63 top-scoring candidates will be prioritized for purification in a heterologous expression system and screened against wheat lines to test for each candidate effector’s ability to produce disease symptoms. We anticipate that P. nodorum effectors that have not yet been identified to the gene level (SnTox2, SnTox4, SnTox5, and SnTox6) may be able to be matched to these candidates using PAV profiles across the sequenced isolates (e.g., SN4, SN79, and Sn99CH 1A7a) to help unmask these recalcitrant loci. Once validated, effector molecules can be applied as tools to accelerate disease resistance breeding programs (Veleshouners and Oliver 2014).

Analyses of the P. nodorum and P. avenae pan-genomes allowed us to quantify different types of genomic variation across a variety of scales. De novo assembly comparisons highlighted the large number of strain-specific loci and the extent of PAV within the two species. Accessory regions were identified, suggesting the possibility of one or more lineage-specific dispensable chromosomes with potential roles in pathogenicity (Zolan 1995; Covert 1998). At the smallest scale of resolution, the pan-genomic comparisons identified loci under diversifying selection. These observations collectively deepen our understanding of the genomic history of Parastagonospora spp. and improve the prediction of potential effector sequences in P. nodorum. We expect that our approach can be broadly applied to other species in the Pezizomycotina, particularly to those which are necrotrophic pathogens.

**Acknowledgments**

This work was supported by access to facilities managed by Bioplatforms Australia; and funded by the Australian Government National Collaborative Research Infrastructure Strategy; Education Investment Fund Super Science Initiative; and Grains Research and Development Corporation research [grant numbers CUR00012, CUR00023, and GRS10061 to R.A.S.]. This work was supported by resources provided by the NCI Specialized Facility in Bioinformatics at The University of Queensland through the National Computational Merit Allocation Scheme; and the advanced computing resources located at the Pawsey Supercomputing Centre supported by the Australian Government.

**Literature Cited**

Abeysekara NS, Friesen TL, Keller B, Faris JD. 2009. Identification and characterization of a novel host–toxin interaction in the wheat–Stagonospora nodorum pathosystem. Theor Appl Genet. 120(1):117–126.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215(3):403–410.

Bankevich A, et al. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 19(5):455–477.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S. 2004. Improved prediction of signal peptides: signalP 3.0. J Mol Biol. 340(4):783–795.

Bertazzi S, et al. 2018. Accessories make the outfit: accessory chromosomes and other dispensable DNA regions in plant-pathogenic Fungi. Mol Plant Microbe Interact. 31(8):779–788.

Buchfink B, Xie C, Huson DH. 2015. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 12(1):59.

Clutterbuck AJ. 2011. Genomic evidence of repeat-induced point mutation (RIP) in filamentous ascomycetes. Fungal Genet Biol. 48(3):306–326.

Covert SF. 1998. Supernumerary chromosomes in filamentous fungi. Curr Genet. 33(5):311–319.

Croll D, Lendenmann MH, Stewart E, McDonald BA. 2015. The impact of recombination hotspots on genome evolution of a fungal plant pathogen. Genetics 201(3):1213–1228.

Croll D, McDonald BA. 2012. The accessory genome as a cradle for adaptive evolution in pathogens. PLoS Pathog. 8(4):e1002608.

Croll D, Zala M, McDonald BA. 2013. Breakage-fusion-bridge cycles and large insertions contribute to the rapid evolution of accessory chromosomes in a fungal pathogen. PLoS Genet. 9(6):e1003567.

Curfer BM. 2000. Stagonospora and Septoria diseases of barley, oat, and rye. Can J Plant Pathol. 22(4):332–348.

Dallery J-F, et al. 2017. Gapless genome assembly of Colletotrichum higginsianum reveals chromosome structure and association of transposable elements with secondary metabolite gene clusters. BMC Genom. 18(1):667.

Emms DM, Kelly S. 2015. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthologous inference accuracy. Genome Biol. 16(1):157.

Friesen TL, Chu C, Xu SS, Faris JD. 2012. SnTox5–SnTox5: a novel Stagonospora nodorum effector–wheat gene interaction and its relationship with the SnToxA–Tsn1 and SnTox3–Snn3–B1 interactions. Mol Plant Pathol. 13(9):1101–1109.

Friesen TL, Meinhardt SW, Faris JD. 2007. The Stagonospora nodorum–wheat pathosystem involves multiple proteinaceous host-selective

**Supplementary Material**

**Supplementary data** are available at Genome Biology and Evolution online.
toxins and corresponding host sensitivity genes that interact in an inverse gene-for-gene manner. Plant J. 51(4):681–692.

Friesen TL, et al. 2006. Emergence of a new disease as a result of inter-specific virulence gene transfer. Nat Genet. 38(8):953–956.

Friesen TL, Zhang Z, Solomon PS, Oliver RP, Fars JD. 2008. Characterization of the interaction of a novel Stagonospora nodorum host-selective toxin with a wheat susceptibility gene. Plant Physiol. 146(2):682–693.

Fudal I, et al. 2009. Repeat-induced point mutation (RIP) as an alternative mechanism of evolution toward virulence in Leptosphaeria maculans. Mol Plant Microbe Interact. 22(8):932–941.

Galagan JE, Selker EU. 2004. RIP: the evolutionary cost of genome defense. Trends Genet. 20(9):417–423.

Gao Y, et al. 2015. Identification and characterization of the StnTox6-SnN6 interaction in the Parastagonospora nodorum–wheat pathosystem. Mol Plant Microbe Interact. 28(5):615–625.

Golicz AA, et al. 2015. Gene loss in the fungal canola pathogen Leptosphaeria maculans. Funct Integr Genom. 15(2):189–196.

Goodvin SB, et al. 2011. Finished genome of the fungal wheat pathogen Mycosphaerella graminicola reveals dispensable structure, chromosome plasticity, and stealth pathogenesis. PLoS Genet. 7(6):e1002070.

Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29(8):1072–1075.

Hane JK. 2015. Calculating RIP Mutation in Fungal Genomes Using RIPCAL. In: van den Berg M, Maruthachalam K, editors. Genetic transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 305(3):567–580.

Hane JK, et al. 2007. Dithiodehydrogenase–plant interactions illuminated by genome sequencing and EST analysis of the wheat pathogen Stagonospora nodorum. Plant Cell 19(11):3347–3368.

Hane JK, Oliver RP. 2010. In silico reversal of repeat-induced point mutation (RIP) identifies the origins of repeat families and uncovereds duplicated genes. BMC Genom. 11(1):655.

Hane JK, Oliver RP. 2008. RIPCAL: a tool for alignment-based analysis of repeat-induced point mutations in fungal genomic sequences. BMC Bioinformatics 9:478.

Hane JK, et al. 2011. A novel mode of chromosomal evolution peculiar to filamentous Ascomycete fungi. Genome Biol. 12(5):R45.

Hane JK, Williams AH, Taranto AP, Solomon PS, Oliver RP. 2015. Repeat-induced point mutation: a fungal-specific, endogenous mutagenesis process. In: van den Berg M, Maruthachalam K, editors. Genetic transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 305(3):567–580.

Ipcho SVS, et al. 2012. Transcriptome analysis of Stagonospora nodorum: gene models, effectors, metabolism and pantothenate dispensability. Mol Plant Pathol. 13(6):531–545.

Jones DA, Bertazzoni S, Turo CJ, Syme RA, Hane JK. 2018. Bioinformatic prediction of plant-pathogenicity effector proteins of fungi. Curr Opin Microbiol. 46:43–49.

Jurka J, et al. 2005. Rebase update, a database of eukaryotic repetitive elements. Cytogenet Genome Res. 110(1-4):462–467.

Kellner R, et al. 2014. Expression profiling of the wheat pathogen Zymoseptoria tritici reveals genomic patterns of transcription and host-specific regulatory programs. Genome Biol Evolut. 6(6):1353–1365.

Krogh A, Larsson B, Von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 305(3):567–580.

Kurtz S, et al. 2004. Versatile and open software for comparing large genomes. Genome Biol. 5(2):R12.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods. 9(4):357–359.

Larkin MA, et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23(21):2947–2948.

Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv Preprint arXiv:1303:3997.

Liu Z, et al. 2009. SnTox3 acts in effector triggered susceptibility to induce disease on wheat carrying the Snn3 gene. PLoS Pathog. 5(9):e1000581.

Liu Z, et al. 2012. The cysteine rich necrotrophic effector SnTox1 produced by Stagonospora nodorum Triggers Susceptibility of Wheat Lines Harboring Snn1. PLoS Pathog. 8(1):e1002467.

Liu ZH, et al. 2004. Genetic and physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-selective toxin produced by Stagonospora nodorum. Phytopathology 94(10):1056–1060.

Ma L-J, et al. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. Nature 464(7287):367–373.

Malkus A, et al. 2005. Sequence diversity of j-tubulin (tub4) gene in Phaeosphaeria nodorum and P.avenaria. FEMS Microb Lett. 249(1):49–56.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. 17(1):10–12. p

McDonald MC, Oliver RP, Friesen TL, Brunner PC, McDonald BA. 2013. Global diversity and distribution of three necrotrophic effectors in Phaeosphaeria nodorum and related species. New Phytol. 199(1):241–251.

McDonald MC, Razavi M, Friesen TL, Brunner PC, McDonald BA. 2012. Phylogenetic and population genetic analyses of Phaeosphaeria nodorum and its close relatives indicate cryptic species and an origin in the Fertile Crescent. Fungal Genet Biol. 49(1):882–895.

Moolhuizen P, et al. 2018. Comparative genomics of the wheat fungal pathogen Pyrenophora tritici-repentis reveals chromosomal variations and genome plasticity. BMC Genom. 19(1):279.

Ohm RA, et al. 2012. Diverse lifestyles and strategies of plant pathogenesis encoded in the genomes of eighteen Dothideomycetes fungi. PLoS Pathog. 8(12):e1003037.

Oliver R. 2012. Genomic tillage and the harvest of fungal phytopathogens. New Phytol. 196(4):1015–1023.

Oliver RP, Friesen TL, Friesen JD, Solomon PS. 2012. Stagonospora nodorum: from pathology to genomics and host resistance. Annu Rev Phytopathol. 50(1):23–43.

Oliver RP, Solomon PS. 2010. New developments in pathogenicity and virulence of necrotrophs. Curr Opin Plant Biol. 13(4):415–419.

Plasson C, Stürchler A, Croll D. 2016. The evolution of orphan regions in genomes of a fungal pathogen of wheat. mBio 7(5):e01231–e01216.

Quaedvlieg W, et al. 2013. Sizing up Septoria. Stud Mycol. 75(1):393–399.

Quinlan AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26(8):841–842.

Quanlin AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26(8):841–842.

Richards JK, Wyatt NA, Liu Z, Faris JD, Friesen TL. 2018. Reference quality accessory chromosomes across isolates of Phaeosphaeria nodorum. J Mol Biol. 199(1):3347–3368.

Rybak K, et al. 2017. A functionally conserved Zn2Cys6 binuclear cluster confers virulence of necrotrophs. Curr Opin Plant Biol. 13(4):393–399.

Shi G, et al. 2015. The wheat gene confers susceptibility on recognition of Zymoseptoria tritici. J Mol Biol. 395(suppl_2):W339–W346.

Syme et al.
Slaton GSC, Birney E. 2005. Automated generation of heuristics for biological sequence comparison. BMC Bioinformatics 6:31.

Smit A, Hubley R. 2010. RepeatModeler Open-1.0. Repeat Masker Website.

Smit A, Hubley R, Green P. 2015. RepeatMasker Open-4.0. 2013–2015. Institute for Systems Biology. http://repeatmasker.org.

Solomon PS, Lowe RG, TAN KC, Waters OD, Oliver RP. 2006. Stagonospora nodorum: cause of stagonospora nodorum blotch of wheat. Mol Plant Pathol. 7(3):147–156.

Stanke M, et al. 2006. AUGUSTUS: ab initio prediction of alternative transcripts. Nucleic Acids Res. 34(Web Server):W435–W439.

Suyama M, Torrents D, Bork P. 2006. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. Nucleic Acids Res. 34(Web Server):W609–W612.

Syme RA, Hane JK, Friesen TL, Oliver RP. 2013. Resequencing and comparative genomics of Stagonospora nodorum: sectional gene absence and effector discovery. G3: Genomes Genes Genetics 3:959–969.

Syme RA, et al. 2016. Comprehensive annotation of the Parastagonospora nodorum reference genome using next-generation genomics, transcriptomics and proteogenomics. PLoS One 11(2):e0147221.

Tan K-C, Oliver RP, Solomon PS, Moffat CS. 2010. Proteinaceous necrotrophic effectors in fungal virulence. Funct Plant Biol. 37(10):907–912.

Testa AC, Hane JK, Ellwood SR, Oliver RP. 2015. CodingQuarry: highly accurate hidden Markov model gene prediction in fungal genomes using RNA-seq transcripts. BMC Genom. 16:170.

Testa AC, Oliver RP, Hane JK. 2016. OccultCut: a comprehensive survey of AT-rich regions in fungal genomes. Genome Biol Evolut. 8(6):2044–2064.

Ueng P, et al. 1998. Intraspecific genetic variation of Stagonospora avenae and its differentiation from S. nodorum. Mycol Res. 102(5):607–614.

Ueng PP, Chen W. 1994. Genetic differentiation between Phaeosphaeria nodorum and P. avenaria using restriction fragment length polymorphisms. Phytopathology 84(8):800–806.

Ueng PP, Cunfer BM, Alano AS, Youmans JD, Chen W. 1995. Correlation between molecular and biological characters in identifying the wheat and barley biotypes of Stagonospora nodorum. Phytopathology 85(1):44–52.

Ueng PP, et al. 2003. Sequence diversity of mating-type genes in Phaeosphaeria avenaria. Curr Genet. 43(2):121–130.

Van de Wouw AP, et al. 2010. Evolution of linked avirulence effectors in Leptosphaeria maculans is affected by genomic environment and exposure to resistance genes in host plants. PLoS Pathog. 6(11):e1001180.

Van De Wouw AP, Howlett BJ. 2011. Fungal pathogenicity genes in the age of ‘omics’. Mol Plant Pathol. 12(5):507–514.

Vleeshouwers VG, Oliver RP. 2014. Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. Mol Plant Microbe Interact. 27(3):196–206.

Zolan ME. 1995. Chromosome-length polymorphism in fungi. Microbiol Rev. 59(4):686–698.

Associate editor: John Archibald